ANNUAL REPORT
OF
PROGRAM ACTIVITIES
NATIONAL INSTITUTE OF
CHILD HEALTH AND HUMAN DEVELOPMENT
FISCAL YEAR 1974
VOLUME II

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service National Institutes of Health

Institutes of Health
a, Maryland 20014

ANNUAL REPORT

OF

PROGRAM ACTIVITIES

NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT

Fiscal Year 1974

Volume II

ADMINISTRATIVE USE

ONLY

ANNUAL REPORT

NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT

July 1, 1973-June 30, 1974

VOL II

OFFICE OF THE SCIENTIFIC DIRECTOR

NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT
ANNUAL REPORT

July 1, 1973 through June 30, 1974

Vol. II

Page

Table of Contents - continued XV

OFFICE OF THE SCIENTIFIC DIRECTOR

Summary Report F-1

Developmental Immunology Branch

Summary Report FA-1

Project Reports:

HD-I-4 Identification and Characterization of Non-pathogenic Bacterial Antigens Cross Reactive With the Capsular Polysaccharides of Meningococcus Groups A, B and C, and Pneumococcal Capsular Polysaccharides Types I and III FA-4

HD-I-8 Identification of the Immune Response to Haemophilus influenzae Type b Capsular Polysaccharide Induced by Feeding of Bacteria With Cross Reacting Antigens to Newborn Rabbits and Primates and Adult Primates and Humans FA-7

HD-I-9 Studies of the Escherichia coli Isolated from Cases of Neonatal Menigitis FA-10

HD-I-10 Isolation, Characterization and Preparation of Diagnostic and Therapeutic Antisera to the Heat Labile Toxin of Escherichia coli Responsible for Enteropathogenic Diarrheal Disease in Infancy FA-13

HD-I-11 Studies on Haemophilus influenzae Type b Polysaccharides FA-15

HD-I-12 Studies on Meningococcal Polysaccharide: Chemical Characterization of the Cross-reacting Antigens from E. coli. FA-17

HD-I-13 Structure and Function of Bacterial Cell Wall: Structure and Immunological Properties of Bacillus pumilis SH-17 FA-20

HD-I-14 Structure and Function of Bacterial Cell Wall: Structure and Biosynthesis of Pneumococcal Cell Wall FA-22

HD-I-15 Studies on Streptococcal Proteinase FA-25

HD-I-16 Methods in Protein Chemistry FA-27

HD-I-17 Physical Properties of Meningococcal and Haemophilus influenzae Type b Polysaccharides FA-30

U556
1974
v. 2

Page

Contract and Collaborative Research:

Characterization of the Development of the Human Infant Immune and Protective Response to Haemophilus influenzae Type b and Neisseria meningitidis Group C Capsular Polysaccharides FA-35

Social and Behavioral Sciences Branch

Summary Report FB-1
  Papers Presented at Professional Meetings FB-5
  Publications FB-6

Project Reports:

HD-SB-10 Environmental Influences on Cognitive and Personality Development in Infancy FB-8
HD-SB-2 An Observational Study of Father-Infant Interaction FB-11
HD-SB-4 Effects of Infant Temperamental Characteristics on Mother-Infant Adaptation FB-13
HD-SB-5 Development of Measures of Social and Motivational Functions in Young Children FB-16
HD-SB-6 Consistency and Antecedents of Infant Attachment During the First Year FB-18
HD-SB-7 Early Environmental Influences on Cognitive and Personality Development During Infancy: A Replication with a Middle-class Sample FB-21
HD-SB-11 Developmental and Situational Factors in Children's Responses to Strangers FB-23
HD-SB-12 Determinants of Infants' Reactions to Strangers FB-25
HD-SB-13 The Measurement of Competence Motivation in One Year Olds FB-28
HD-SB-14 Early Parent-Infant Interaction and Cognitive Development Among Premature Infants FB-31

Reproduction Research Branch

Summary Report FC-1
  Clinical Investigations FC-2

Project Reports:

HD-RPL-1 Biology of Reproduction: Clinical and Experimental FC-4
HD-RPL-11 Mechanism of Action of Protein and Polypeptide Hormones FC-15
HD-RPL-5 Analytical Techniques and Instrumentation
  a. Amino Acid Analysis
  b. Automatic Monitoring of Chromatographic Experiments FC-19
HD-RPL-6 Synthesis of Biologically Active Peptides FC-22
HD-RPL-7 Cell Organelles from Human Placenta: Formation, Isolation, Characterization, and Function Under Normal and Abnormal Conditions FC-25
HD-RPL-9 Chemical Modification of Amino Acids. Selective Nonezymatic Cleavage of Peptide Bonds. Structure-Function Relationship of Biologically Active Peptides and Enzymes FC-27

Page

HD-RPL-10 The Physical Chemistry of Membrane-active Peptides. Peptides with α, B-Unsaturated Amino Acids and Lanthionines and Their Effect on Neonatal and Neoplastic Tissue FC-30
HD-RPL-12 Structure and Function of Proteins FC-32
HD-RPL-13 Application of Stable Isotopes to Biochemical Problems FC-34
HD-RPL-14 Binding Studies of Ligand or Hormones Using NMR FC-35
HD-RPL-2 Testicular Factors Regulating Secretion of Pituitary Gonadotropins (1973 title: "Physiology of Testis") FC-37
HD-RPL-3 Fertilization and Embryogenesis FC-39
HD-RPL-4 Hormonal Interrelationships Between Fetus, Mother and Placenta Throughout Gestation in Rhesus Monkeys (1973 title: "The Use of Rhesus Monkeys in Studies of Pregnancy") FC-40
HD-RPL-15 Measurement of Chorionic Gonadotropin in the Rhesus Monkey and Its Significance in Early Pregnancy FC-43

Honors and Awards FC-45
Invited Lectures FC-45
Publications FC-48

Behavioral Biology Branch

Summary Report FD-1
Section on Neurobiology FD-1
Section on Functional Neurochemistry FD-3
Section on Brain and Behavior FD-5

Project Reports:

HD-BB-4 Information Processing in the Central Auditory System of Mammals FD-7
HD-BB-5 Neurobiologic Studies of Neuronal and Other Cell Types in Cell Culture FD-10
HD-BB-8 Correlative Neurochemical and Electrophysiological Analyses of Individual, Identifiable Neurons in Molluscan Nervous Systems FD-15
HD-BB-9 A Correlated Study of the Fine Structure and the Function of the Endoplasmic Reticulum (ER) of Neurons FD-21
HD-BB-10 Changes in Membrane Properties and Fine Structure Associated with Cell Morphology FD-24

Laboratory of Molecular Genetics

Summary Report FE-1

Project Reports:

HD-LMG-5 Control Mechanisms in Temperate Bacteriophage λ FE-6
HD-LMG-1 Integrative Control of Macromolecular Synthesis FE-10
HD-LMG-2 Factors Influencing Genetic Transcription-Initiation and Termination FE-16

Page

HD-LMG-8 Molecular Aspects of the Replication of Enveloped Animal RNA Viruses FE-19
HD-LMG-9 Morphogenesis of Animal Viruses During Infection of Mammalian Cells FE-22
HD-LMG-4 Study of Adenovirus Gene Functions FE-25
HD-LMG-3 Molecular Aspects of the Differentiation of Embryonic Chick Lens Fibers *in vivo* and in Tissue Culture FE-27
HD-LMG-7 Regulation of Antibody Biosynthesis FE-32
HD-LMG-6 Regulated Expression During Cell Growth and Differentiation FE-35

Laboratory of Biomedical Sciences
Summary Report FF-1

Project Reports:
HD-LB-1 Developmental Enzymology FF-5
HD-LB-9 The Effect of Metabolic Disorders on the Biochemistry, Structure, and Function of the Developing Brain FF-8
HD-LB-10 Mechanism of Aromatic Hydroxylation FF-11
HD-LB-11 Biosynthesis and Function of Pteridines FF-13
HD-LB-12 Biochemistry of Brain Macromolecules FF-15
HD-LB-4 Development of Mammalian Metabolic Regulation FF-17
HD-LB-18 Regulation of Neuroendocrine Metabolism FF-20
HD-LB-8 Developmental Pharmacology FF-23

Pregnancy Research Branch
Summary Report FG-1

Project Reports:
HD-PR-1 Carbohydrate Metabolism During Primate Pregnancy FG-5
HD-PR-2 Regulation of Estrogen Synthesis in Pregnant Baboons FG-9
HD-PR-3 Amniotic Fluid Lecithin Synthesis in Primate Pregnancy FG-12
HD-PR-5 Calcium Homeostasis in the Subhuman Pregnant Primate FG-14
HD-PR-7 Renin-Angiotensin Relationships in Sheep Pregnancy FG-16
HD-PR-8 Thermo Regulation in the Pregnant Sheep FG-18
HD-PR-9 Placental Mechanisms for Regulation of Steroid Hormone Synthesis in Primate Pregnancy FG-20
HD-PR-10 Biochemical Indices of Feto-Placental Status in Human Pregnancy FG-22
HD-PR-11 Cortisol Metabolism in the Baboon During Pregnancy FG-24

Page

GERONTOLOGY RESEARCH CENTER

Office of the Chief

Summary Report FH-1
Technical Development Section FH-1
Photography and Arts Section FH-1
Collaborative Guest Scientist Program FH-3
Intramural Research FH-12

Laboratory of Behavioral Sciences

Summary Report FI-1

Project Reports:

HD-LBS-1 Behavioral Genetics and Aging FI-4
HD-LBS-2 Daydreaming and Aging: Normative and Experimental FI-10
HD-LBS-3 Learned Modification of Visceral Function in Animals FI-24
HD-LBS-4 Problem Solving and Age FI-32
HD-LBS-5 Verbal Learning and Age FI-39
HD-LBS-6 Perceptual Retention and Age FI-47
HD-LBS-7 Reaction Time and Age FI-51
HD-LBS-8 Learned Modification of Visceral Function in Man FI-53
HD-LBS-9 Operant Performance, Memory and Aging FI-59
HD-LBS-10 Exercise, General Activity Level and Aging FI-64

Clinical Physiology Branch

Summary Report FJ-1

Project Reports:

HD-CP-7 The Kinetics of Glucose and Insulin Metabolism in Man FJ-7
HD-CP-20 Uremia and Carbohydrate Intolerance FJ-10
HD-CP-12 Cytoxan-induced Water Intolerance FJ-12
HD-CP-27 Effect of Age on Metabolism FJ-13
HD-CP-30 The Effect of Age on the Pharmacokinetics and Pharmacologic Effects of Ethanol in Man FJ-19
HD-CP-17 Effect of Age on the Response to Inotropic Interventions and Hypoxia of Rat Trabeculae Carneae FJ-21
HD-CP-31 Hemodynamics of the Left Ventricle and Mitral Valve in Aging Man FJ-23
HD-CP-18 Hormone Receptors and Aging: Effects of Age on Hormone Mediated Alterations of Adenylate Cyclase, Tissue Cyclic AMP and Factors Controlling These Metabolic Regulators FJ-25
HD-CP-28 The Biochemistry of Renin FJ-28
HD-CP-23 Longitudinal Studies of Human Physiology, Biochemistry and Psychology FJ-30
HD-CP-24 Age Changes in Human Performance FJ-33
HD-CP-25 Age Relationships of Body Composition, Nutrition and Physical Activity FJ-37
HD-CP-22 Marital, Sexual and Social Factors in Aging FJ-40

Page
HD-CP-13 Dermatoglyphics and Clinical Medicine FJ-43
HD-CP-15 Dermatoglyphics of Primitive and Other Populations FJ-45
HD-CP-14 Twin Studies: I. Structure and Color of the Iris
II. X Chromosome Inactivation
III. Dermatoglyphics and Zygocity Determination FJ-48
HD-CP-16 Genetic Studies on Longitudinal Program Subjects FJ-50

Laboratory of Cellular and Comparative Physiology

Summary Report FK-1

Project Reports:

HD-CCP-1 Changes in Cells and Extracellular Matrices of the Crayfish Gastrolith Disc During Growth, Development and Aging FK-4
HD-CCP-2 Dietary Proteins and Longevity FK-5
HD-CCP-8 Age-related Changes in Mammalian Myocardial Cells FK-6
HD-CCP-11 Ultrastructural Studies of the Regenerating Sea Urchin Spine During Growth and Developmental Sequences FK-9
HD-CCP-12 Cellular Basis of Regulation of the Humoral Immune Response FK-10
HD-CCP-15 Cellular Aspects of the Immune Response of Long-term Radiation Induced Allogeneic Chimeras FK-13
HD-CCP-16 *In vitro* Studies on the Capacity of Aged Mice to Develop Immunological Memory FK-17
HD-CCP-17 Pathology of Age-associated Kidney Disease of Wistar Rats Reared at GRC FK-19
HD-CCP-18 Effects of Age on Steroid Hormone Binding and Responsiveness in Target Cells and Tissues FK-22
HD-CCP-19 Mechanisms of *in vitro* Cellular Ageing (formerly entitled "Effect of Chromosomal Aneusomy on Cellular Growth, Metabolism and Aging") FK-25
HD-CCP-20 Effects of the Agouti Locus on Development and Aging in Mice FK-29
HD-CCP-21 The Use of Dietary Probes to Study Mechanisms of Age Related Immunological and Biochemical Functions (formerly entitled "Effect of Dietary Manipulations on Life Span, Incidence of Diseases and Immunologic and Related Functions") FK-31
HD-CCP-22 Ageing and Genetically Defective Mammalian Skeletogenic Cells and Connective Tissues FK-35
HD-CCP-23 Low Temperature Effects on Cells of Ageing Individuals (formerly entitled "Studies on the Comparative Physiology of Ageing and Environmental Effects on the Ageing Process") FK-36

Page
HD-CCP-24 Effects of Aging on the Modulation of Proliferation and Differentiation of Immunocompetent Cells (formerly the two following projects "Characterization of Age-related Defect in the Proliferative Capacity of Lymphocytes" and "Characterization of Cellular Deficiencies in Age-related Decline of the Humoral Immune System") FK-39
HD-CCP-25 The Role of Cell Membrane Structures in Cellular Recognition Phenomena FK-44
HD-CCP-26 Mechanisms of the Maternal Age Effect as Expressed by an Increased Frequency of Chromosomal Aneuploid Offspring with Increased Maternal Age FK-47
HD-CCP-27 Characterization of the Immune System of Aging with Immunodeficiency Diseases FK-49
HD-CCP-28 Conscription and Polyadenylation of Genetic Messages During Early Embryogenesis FK-51

aboratory of Molecular Aging

Summary Report FL-1

Project Reports:

HD-GMA-3 Structure of Nucleoprotein, Ribosome, and Interactions of Components Related to Protein Synthesis FL-6
HD-GMA-5 Polymers as Biological Reagents FL-9
HD-GMA-9 Relation of Structure and Function in Hemoglobin FL-12
HD-GMA-12 Effects of Metal Ions on the Structure and Function of Nucleic Acids FL-15
HD-GMA-13 Control of Intermediary Metabolism: Biology of Membranes FL-20

NICHD ANNUAL REPORT

July 1, 1973 through June 30, 1974
Office of the Scientific Director

Important scientific research accomplishments continued to flow from the laboratories of the NICHD intramural program in fiscal year 74 as scientists continued research efforts in maternal and child health, reproduction, and aging. Highlights of their accomplishments are described in the accompanying reports and are summarized below. To foster these research programs new facilities have been planned, designed, and constructed. A new temporary building designed to permit surgery and research on pregnant sheep and primates has been completed, and scientists in the Pregnancy Research Branch have begun using this facility, probably the finest in the world for this type of research. Construction is well under way on the Perinatal Biology facility in the NIH Clinical Center with occupancy scheduled early in fiscal year 75. Scientists have been recruited to initiate the research program in pregnancy and neonatology that this facility will permit. Plans have been completed for a temporary addition to Building 6 to provide space to expand the Laboratory of Molecular Genetics and the Laboratory of Biomedical Sciences. Plans for Building 33, the NICHD Building, are complete and wait only budgetary appropriation for construction.

In fiscal year 1974 total budgeted positions for intramural research increased from 321 to 322. In addition, a total of 117 scientists worked in NICHD laboratories as Guest Workers, Guest Scientists, and Visiting Fellows. Another 58 employees in part-time, temporary, WAE, Stay-in-School, and Work Study categories participated in the intramural research programs.

Developmental Immunology Branch - Accomplishments

1. A clinical trial is under way in Mecklenberg County, North Carolina of a new vaccine for Hemophilus influenzae type b. In this trial all infants appearing for their routine immunization program at the offices of private pediatricians, general practitioners, county health department stations or the out-patient clinic at the Charlotte Memorial Hospital are offered vaccination with either the Hemophilus influenzae type b vaccine or meningococcal group c vaccine, in a blind randomized fashion. It is estimated that this study will provide an assessment of the vaccine's protective effect in children in 2 to 3 years.

2. Another means of immunization against Hemophilus influenzae type b is also under investigation. Animal studies have demonstrated that feeding newborn rabbits or primates with specific serotypes of either E. coli or B. pumilis stimulates the production of effective antibody levels against H. influenzae as soon as passively acquired maternal antibody levels decline. Further, the newborn primates have shown no adverse reactions to doses of as high as 10^{11} organisms fed at birth.

Continued studies of infants colonized spontaneously while in the newborn nursery with a strain of *E. coli* having cross-reacting antibodies to *Haemophilus influenzae* type b indicate that these infants develop antibodies to *H. influenzae* more rapidly and in higher titers than do their age matched controls who are not colonized in the nursery. Six adults have been successfully colonized with 10^8 *E. coli* strain Easter which has cross-reacting antigens to *H. influenzae*. Results so far indicate that the adults respond with a 2 to 3-fold increase in serum antibodies to *H. influenzae* type b. These studies are continuing. In addition, the structural basis for the cross-reactions between the capsular polysaccharide of the organisms is also under intensive study.

Social and Behavioral Sciences Branch - Accomplishments

1. The role of the father in influencing early child development has been little studied, with most emphasis placed on maternal-child interaction. A study just completed by this Branch has examined the father's influence on young infants, both directly and indirectly as mediated through the mother. Analysis of the data indicates that paternal attitudes influence the mother-son relationship, but have no effect on the mother-daughter relationship. One area of the mother-son relationship that seems to be especially vulnerable to the father's influence is her handling of feeding. Disruptive patterns in the mother's care of male infants often reflect conflict in the marital relationship. These examples of the findings emphasize the importance of considering the interaction of both parents with the infant and with each other in studying the early environment.

2. Another interaction that has been little studied in child development is the child's role in determining interaction with his mother. Consequently, a study was undertaken in this Branch to assess the effects of the infant's temperament characteristics on mother-infant interaction. In an attempt to determine how the infant's sensitivities and early characteristics mediate stimulation and responses from the mother, familes were studied during the last trimester of pregnancy, the infant's neonatal characteristics were evaluated and mother-infant interaction was assessed at 4 weeks and 6 months. While this study is not yet complete, some important observations have emerged. Women who felt they received adequate support during pregnancy and found their labor and delivery experience favorable and received lower doses of anesthesia all tended to have positive self-images. They expressed confidence in their ability to function effectively in the maternal role, their capacity to relate maturely to others, and in their feelings of self-worth. Also found was a significant relationship between the analgesia and the anesthesia administered during labor and delivery, and some characteristics of infant temperament at 3 days. Use of local or regional anesthetics correlated with babies motor immaturity, irritability, and diminished attentiveness; and analgesia dosage correlated with motor maturity. The woman's adaptation to pregnancy was related to whether or not she received delivery medication. These findings suggested there is an interaction between prenatal and perinatal influences in the behavior of newborn infants.

3. Another study in the Branch has called into question the generalization that fear of strangers is a normal aspect of child development

in the second half of the first year. Findings in this study indicate that infants' reactions to unfamiliar adults vary considerably depending upon their age, proximity of the mother, and specific behavior of the stranger. The infant's response to strangers was found to be unrelated to experiential or temperamental variables, but rather seemed to be a situationally determined response. Analysis of the infant's behavior towards strangers and the mother in different situations suggests that crying behavior may be more a form of communication than a manifestation of fear.

Reproduction Research Branch - Accomplishments

1. Basic research in laboratories in this Branch has provided a major advance in the field of protein hormone synthesis, an area of great significance and interest in reproductive biology at the present time. Scientists in the Branch have described a new solid phase method for synthesis of peptides via alpha, beta-unsaturated amino acids which offers significant advantages over current methods. This work derives from studies of such amino acids as normal constituents of several membrane-active antibiotics. The identification of these unusual amino acids opens avenues for research in targeting synthetic peptides for specific membranes.

2. The adrenal medulla is being intensively studied by scientists in the Branch as a model of a hormone secretory system. By special electron microscopy techniques secretory vesicular membranes were shown to fuse with plasma membranes, presumably as part of the secretory process. Activation of adenyl cyclase in these isolated membranes by agents such as carbachol suggests that activation is related to the secretory process.

3. Another major thrust of the Branch has been study of the gonadotropins. In the past year the beta sub-unit of human chorionic gonadotropin (hCG) has been isolated totally free of contaminants by polyacrylamide gel electrophoresis. Subsequently, the carboxy-terminal amino acid sequence unique to hCG beta subunits was used to immunize rabbits. Resulting antiserum had complete specificity for hCG. The immunoassay system is presently being developed so that it may be used eventually as an office test for diagnosing pregnancy. Its sensitivity will be such that pregnancy can be diagnosed 8 to 10 days after conception, which is 4 to 6 weeks sooner than possible by conventional office procedures. Similarly an immunoassay system for monkey chorionic gonadotropin has been developed. This test will facilitate studies in primates since pregnancy can be diagnosed 18 days after fertilization.

4. Amenorrhea and infertility are being investigated by other scientists in the Branch. Two types of patients with secondary amenorrhea have been distinguished on the basis of the response of their gonadotropins to clomiphene citrate and LHRH. This differentiation will permit more rational therapy for infertility. Other studies have demonstrated that some amenorrheic women also have aberrations of core temperature control, and abnormalities of ADH release. This is the first evidence of hypothalamic disturbance in amenorrhea. Infertility studies in men

ave demonstrated that FSH is not useful in treating men with oligospermia n contrast to previous reports by other researchers. In addition, electron roscopy studies have provided the first anatomic evidence of a Sertoli cell disorder in a number of infertile men.

Behavioral Biology Branch - Accomplishments

Section on Neurobiology

1. Additional advances have been made in the past year in the study of nerve cells in tissue culture. Well differentiated neurons from fetal rat brain have been grown by a special culture method involving use of fluorodeoxyuridine and horse serum. These cells have been characterized and categorized on the basis of their morphology in the living state and their appearance on silver staining as well as their electrical excitability and response to neurotransmitters. These cells are being compared to those grown in the absence of fluorodeoxyuridine to examine biosynthesis of transmitter-related enzymes. Cells from the cerebral cortex and other parts of the brain can be ultured independently so that regional studies of the brain can be undertaken, and specific neuronal populations studied.

2. Evidence has been obtained for the first time for biochemical interaction between spinal cord cells and muscle cells in tissue culture. Activity of the enzyme choline acetyltransferase in nerve cells is augmented by greater than 10-fold when these cells are co-cultured with muscle cells. The enzyme is responsible for the synthesis of acetylcholine which is the transmitter at the myoneural junctions, so the interaction has functional significance. When the medium in which the muscle cells have been grown is added to the nerve cells in culture, a partial effect follows so some diffusible substance appears to be involved. Experiments are in progress to determine its nature. Enzymes other than choline acetyltransferase are changed very little when nerve is co-cultured with muscle, so the effect appears specific.

3. Light microscopic time lapse photography has been used to study transport of materials within axons and cellular processes. By this method particles can be seen to move. Observations have been made of this process in the spinal cord, brain and dorsal root ganglion cells, in various types of fibroblasts, and neuroblastoma cells. The rate of movement of the particles is 1 to 3 microns per second. Disruption of the microtubules or increased intracellular calcium concentration reduce or abolish this particle movement.

Section on Functional Neurochemistry

Scientists in this section have continued their studies of the neurosecretory process in single neurons from a large land snail. Their studies have demonstrated an important regulatory role for divalent cations both in the seasonal modulation of the neurosecretory cell's rhythm and in the bursting pacemaker potential of the cell. Consequently, study of hormonal effects on electrophysiology and protein synthesis patterns of specific neurons was pursued more intensively. It was found that aldosterone as well as other corticosteroids inhibit bursting pacemaker activity whereas vasopressin dramatically augments such activity in a specific manner. A specific active peptide fraction from the brain of the animal studied behaves similarly to vasopressin. Correlation of the morphological changes in neurosecretory neurons with electrical, metabolic, and secretory activity has demonstrated changes dependent on calcium concentrations. These studies reinforce the conclusion that the nervous system of the mollusk studied represents an excellent model of hypothalamic regulatory systems. The neuron studied is modulated by dopaminergic synaptic input, and receives long-term negative feedback via circulatory steroids, and positive feedback by small peptides. This single neuron possesses the wide range of phenomenology characteristic of hypothalamic nuclei and is amenable for detailed study.

Section on Brain and Behavior

Efforts of scientists in this section continue to be devoted to analysis of monkey vocalization. Computer programs have been developed to permit large scale processing of the vocalizations for analysis. Studies will focus on possible genetic basis of structural differences in the calls as well as individual specificity. Concomitantly, the neurophysiological and functional properties of a major neural pathway from the lateral frontal cortex to the temporal lobe are being studied. It is thought that this pathway may play a role in processing complex auditory input. Single unit recordings from monkeys are being utilized to study influence on auditory cortex neurons of electrical stimulation of the lateral frontal cortex. Experiments are also under way to establish neuropharmacological mechanisms underlying unit responsiveness to vocalization.

Laboratory of Molecular Genetics - Accomplishments

1. Scientists in this Branch have made considerable progress in studies directed toward understanding the organization of antibody genes. By purifying an immunoglobulin mRNA corresponding to a mouse kappa light chain, and using it as a template for reverse transcriptase, synthetic DNA complementary to this message has been obtained. This DNA has been used to distinguish between several models advanced to explain the diversity of antibody structure. Results indicate that the genetic sequence corresponding to the constant region of the immunoglobulin light chain is represented probably less than 3 times per haploid genome. A stringent type of germ line hypothesis advanced to explain the organization of immunoglobulin genes is thus ruled out. The results require postulation of a recombination step which, either at the level of the gene or the message, would serve to bring together both variable and constant regions of the light chain. Future studies with this system will be directed to distinguishing between the possibilities that antibody diversity has arisen through evolution or repeatedly arises during somatic differentiation of immunocytes.

Regulatory pathways affecting antibody biosynthesis at the cellular level have been studied by other scientists in the Branch. Efforts have been directed to understanding the role of T-cells in enhancing or suppressing antibody secretion and proliferation of B-cells. It was found that streptococcal exotoxin serves as a strong immunosuppressant and that, after treatment with this agent, recovering animals had markedly elevated antibodies. Utilizing mice which congenitally lack a thymus, it was found that streptococcal exotoxin failed to suppress antibody levels and did not enhance antibody production after treatment. These results suggest that the effect of this protein exotoxin is at the level of the T-cell.

Laboratory of Biomedical Sciences - Accomplishments

Section on Developmental Enzymology

Changes in activities of two catecholamine-catabolizing enzymes, catechol-o-methyltransferase (COMT) and monoamine oxidase (MAO), in rat placenta during pregnancy showed that both increased significantly during the latter part of gestation. MAO, but not COMT, decreased significantly between the 20th day and birth. In human placenta the mean COMT activity at term was half that of rat term placenta, where as MAO activity of human placenta was 5 times that of the rat. The enzyme activities were also studied in choriocarcinoma cells in tissue culture. MAO activity was absent, and only slight COMT activity was present.

Section on Intermediary Metabolism

A number of enzymes important in intermediary metabolism of phenylalanine have been studied and characterized. GTP-cyclohydrolase, the first enzyme in the pteridine biosynthetic pathway, was purified to homogeneity and its product was identified as D-erythro-neopterin triphosphate. Bacterial tryptophan hydroxylase and dihydropteridine reductase were also purified. Phenylalanine hydroxylase was studied and its mechanism of control by product and product metabolites was elucidated.

Section on Physiological Controls

Studies in this section have focused on the synthesis and mode of action of cyclic AMP. In study of the glycogen cycle enzymes a phosphoprotein phosphatase has been identified and purified which specifically reverses the effects of cyclic AMP-dependent protein kinase. In addition, phosphorylase phosphatase has been found to be a third enzyme in glycogen metabolism which is controlled (inactivated) by the cyclic AMP-dependent kinase.

A method was developed for culturing fetal liver cells free of fibroblasts in order to study glycogen synthesis. Using this system it was found that adrenal glucocorticoids specifically initiate the developmental appearance of glycogen synthetase in these cells.

Section on Developmental Pharmacology

1. Using inbred strains of mice, it has been determined that induction of at least 8 drug metabolizing enzyme activities segregates as a single autosomal dominant trait. It has also been found that a new hemoprotein, cytochrome P_1 450, is always concomitantly synthesized during the induction process. Mice whose enzyme activities are relatively unresponsive to induction by aromatic hydrocarbons can become responsive if a compound having an extremely high avidity for all tissues is administered. This suggests that the non-responsive mice have all the necessary regulatory and structural genes necessary for induction of these enzyme activities, but that the difference reflects a genetically defective receptor binding-site for the aromatic hydrocarbon inducers in the nonresponsive mice.

2. An experimental model for studying enzyme induction has been developed using fetal cell cultures derived from the entire hamster or mouse or from liver. Using this model it was found that of two possible forms of aryl hydrocarbon hydroxylase activity found in the intact animal, only one form was induced in fetal liver cell culture regardless of the type of inducer used in the medium. These results indicate that extrapolation of data from cell culture to the intact animal is not always possible.

Pregnancy Research Branch - Accomplishments

1. Scientists in this Branch are continuing to use animal models including the Rhesus monkey, baboon, and sheep to study physiology and biochemistry of normal and abnormal pregnancy. Calcium homeostasis has been studied in monkey pregnancies. There is no detectable parathormone in fetal plasma. Reduction of fetal ionized calcium levels by infusion of EDTA directly into the fetus does not cause elaboration of parathormone by the fetus; but maternal serum parathormone levels rise for reasons that are not clear. On the other hand, when maternal ionized calcium levels are decreased by EDTA infusion to the mother, fetal serum ionized calcium levels do not change. Maternal parathormone levels rise, but no fetal parathormone is detectable. After birth, parathormone is detectable in the fetus from 12-48 hours after birth. Challenge of the newborn with EDTA induced hypocalcemia causes a prompt increase in serum parathormone levels. Investigations are continuing in an attempt to understand the absence of parathormone in the fetus and its prompt appearance after birth.

2. Monkey models have also been used to study amniotic fluid lecithin synthesis. The lecithin sphingomyelin ratio increases sharply when 92 to 94% of gestational has been completed in the monkey just as it does in the human, thus providing a marker of fetal maturity. This has been used in the human to determine when a fetus is mature enough that it can be delivered without risk of hyaline membrane disease. Studies using the monkey model have demonstrated that only the choline pathway of lecithin synthesis is active, just as in humans. The fact that the monkey model is consistent with the human data will now

use of the monkey to study premature induction of lecithin biosynthesis using corticosteroids, to provide a more detailed analysis of this technique which has been shown effective but of unknown risk in human subjects.

. Efforts are continuing to develop and improve biochemical methods of assessing feto-placental function in high risk pregnancies. Studies in this Branch have shown that hormones are cleared far more rapidly than enzymes from maternal blood, and thus provide an earlier means of detection of feto-placental compromise than would enzyme administration. Work is focusing on intra-amniotic administration of dehydroepiandrosterone sulfate to women in the third trimester, which produces significant increases in maternal blood and urinary estrogen levels. Serum estradiol levels showed the most rapid response; thus assay of serum estradiol after intra-amniotic administration of dehydroepiandrosterone sulfate may provide a convenient, sensitive and clinically useful index of placental functional capacity.

GERONTOLOGY RESEARCH CENTER

Laboratory of Behavioral Sciences - Accomplishments

1. Much controversy exists as to whether intellectual ability declines with age. Psychometric tests of verbal intelligence indicate no decline with age, even with patients in their 70s and 80s. Tests by scientists in this Branch of patients in the longitudinal study have demonstrated that some deficits in performance do appear with age, depending on the specific task involved. The performance of older men followed longitudinally declines markedly with age when the task requires them to process new information. These deficits are observed in the acquisition, retention, and utilization of this new information. It is suggested that the difference between these studies of intellectual function and psychometric studies is that the latter assess the degree to which subjects can retrieve old, well-learned information, whereas the present studies are assessing ability to process new information.

2. Important differences have also been noted in perceptual functioning of subjects as they age. Marked age deficits have been detected in studies of dichotic listening (appreciation of verbal information simultaneously presented to each ear separately). Young men generally have no difficulty repeating different sets of up to 4 digits presented dichotically. Men over 60 are unable to report 1 of the sets of even 2 digits so presented. The mechanism is clearly not related to changes in memory since these men over age 60 can easily recall 6 or more digits when presented serially and not dichotically. Other studies in the laboratory have shown that older men are highly susceptible to effects of interference in learning. The dichotic effect may be another manifestation of this phenomenon. These studies represent a powerful tool for assessing the mechanism by which the brain processes and classifies new information.

3. In a series of clinical studies scientists in the Branch have demonstrated that an operant conditioning procedure is effective in eliminating

fecal incontinence in either young or old subjects. The simplicity of this approach, the lack of effective alternatives for this distressing disorder, and the high success rate of operant conditioning suggest that this method should now be the therapy of choice for the condition.

4. Operant conditioning studies have also been employed to train patients to control their systolic blood pressures and their heart rates. Studies of the mechanism of this response have now been completed. The decrease in blood pressure is accomplished by modifying peripheral resistance. Studies of control of heart rate in animals have shown that parasympathetic blockade prevents increasing heart rate, but ability to slow the heart rate is unimpaired. Sympathetic blockade prevents reducing heart rate, but does not affect speeding of the rate. Total autonomic blockade prevents the animals from changing their rates in either direction. These results indicate that heart rate slowing is mediated by withdrawal of beta sympathetic stimulation and heart rate speeding is mediated by vagal withdrawal.

Clinical Physiology Branch - Accomplishments

1. Subjects in the Longitudinal Study of Aging have provided a valuable and useful population for study of a number of functions in relation to age. In one study utilizing these subjects, the renal response to moderate water deprivation was investigated. All subjects were free of renal disease, diabetes, coronary or cerebrovascular disease and were receiving no medications affecting renal function. Measurements of urine flow and osmolality were obtained at 3, 6, 9 and 12 hours after the onset of water deprivation. Initially there were no significant age differences in urine flow or osmolality. However, data from the last 6 hours of water deprivation showed a significant, positive correlation between age and urine flow, and a significant negative correlation of age with urine osmolality. The degree of impairment with age in ability to decrease urine flow was greater than the inability to concentrate urine, so osmolar clearance actually increased with age. This age-related impairment in fluid and electrolyte homeostasis is probably related to the predisposition to volume depletion of elderly subjects.

2. In another study utilizing subjects in the Longitudinal Study, the drug antipyrine has been administered intravenously to estimate total body water over the past 14 years. In addition to the total body water estimates, the drug measurements were used to estimate plasma half-life of the drug. In the 516 subjects studied there was a highly significant decrease in half-life with age. The younger subjects (20-39 years) show a half-life 39% greater than the older subjects (60-79 years). Since this is a marker drug for microsomal enzyme degradation, these data on drug kinetics and changes with age are of great interest.

3. Previous studies of the subjects in the Longitudinal Study demonstrated a striking decrease in pyridoxal phosphate (Vitamin B6) plasma levels with age. Consequently, computer analysis was done of one-week dietary diaries maintained by the subjects in order to estimate

dietary intake of Vitamin B6. Decreased intake of the Vitamin was found. The oldest subjects averaged only 1.27 mg intake per day compared to 1.77 mg per day for the youngest subjects. These mean values compared to the NRC recommended allowance of 2 mg per day. Six percent of the subjects who were not taking supplementary vitamin preparations were taking in less than 1 mg per day and over all 72% were below the recommended allowance. These data are of particular interest since the subjects are an educationally advantaged group and would be expected to have an above average nutritional state.

Laboratory of Cellular and Comparative Physiology - Accomplishments

1. Scientists in this Branch have been studying intensively the changes in cellular and functional properties of some subpopulations of lymphoid cells with age. In the past year scientists in the Branch have observed that the age-related decline in DNA synthetic activity of T-cells correlates most strikingly with the level of cyclic GMP. Changes in the level of cyclic nucleotides can be detected within minutes after the cells are stimulated by mitogens and long before the on-set of DNA synthesis. Further, there is evidence that these changes in the intracellular levels of cyclic nucleotides are responsible for initiation and regulation of cellular proliferation and differentiation. Consequently, it is felt that the major changes responsible for these effects are probably occuring at the membrane level.

2. Studies are continuing on the effects of aging on the modulation of proliferation and differentiation of immunocompetent cells. In experiments conducted this year young mice were made deficient in T-cells by various means. Thymic epithelial cells of either old or young mice were then transplanted into these T-cell deficient young mice. The cells from the older mice were incapable of transforming precursor cells into functional T-cells in contrast to the ability of the cells from the young mice to perform this function. Studies are now in progress to determine whether this age-related defect may be reversible.

3. Maternal age is known to increase the frequency of chromosomally abnormal offspring. Studies have been initiated using the mouse as a model to study this effect of maternal age. The mouse model thus far seems to simulate the human condition. Increased numbers of chromosomally aneuploid offspring were obtained from older mice. Work with this model will continue.

4. Tissue culture studies of the Hayflick phenomenon revealed a striking difference in various cytokinetic indices between lung and skin fibroblasts obtained from the same embryo. The lung fibroblasts had indices similar to early passaged Hayflick WI-38 (lung) cells, whereas those from skin resembled late passaged WI-38 cells. This finding emphasizes the danger in equating lung and skin fibroblasts, as has been commonly done in the past, and suggests caution in use of some in vitro population aging parameters as a reflection of functional aging at the individual cellular level.

Laboratory of Molecular Aging - Accomplishments

1. Studies have continued this year utilizing the renal brush border membrane as a model for studying transport of substances in the kidney. Previous studies had delineated the mechanism of glucose transport. Studies this year focused on transport of amino acids. It was shown for the first time that uptake of an amino acid (alanine) by the isolated membrane was consistent with the concept of Na+ gradient dependent facilitated transport across the luminal membrane. Na+ was also found to stimulate gamma-glutamyl transpeptidase activity. Activity of this enzyme was concentrated in the purified brush border membrane. It is likely that this enzyme is the amino acid carrier for transport across the membrane.

2. Metal ions have been used to probe the mechanism of transcription of RNA polymerase. Geometrical isomers of a platinum compound were used to modify the DNA template by complex formation. Results indicated that the positions of the Pt atoms in a complex with the DNA template could specifically serve as message limiting loci. RNA polymerase activities stopped whenever a geometrically restricted base sequence was reached. These metal complex probes have significant potential usefulness in studying DNA structure and relationship to message coding.

3. Vinyl analogs of polynucleotides have been found in previous studies to offer protection to cells against infection by viruses. Studies in this year have assessed efficacy of these vinyl analogs in protecting cells against infection by leukemia virus. Studies showed that the analogs had only a slight toxicity to cells in tissue culture and to mice in vivo and protected both of them against infection by murine leukemia virus, but not against several other tumor viruses. These analogs inhibited the reverse transcriptase activity characteristic of tumor viruses. Thus, an effect has been demonstrated in addition to their action in inducing interferon production.

Collaborative Guest Scientist Program - Accomplishments

1. Alterations in the mechanical performance of cardiac muscle with age have been studied utilizing isolated heart muscle from young and old rats. Dose-response curves to inotropic stimulation with calcium did not change with age, suggesting that the ability of the contractile element of muscle to respond to inotropic stimulation by calcium is not modified by aging. The age associated decrease in inotropic response to catecholamines is not due to an increased uptake of norepinephrine by nerve endings, since the depression of inotropic response in old muscles was similar whether stimulated with norepinephrine, which is taken up by nerve endings, or with isoproterenol, which is not taken up by nerve endings. The decrease thus appears related to an age-associated alteration in the response sequence along the catecholamine cascade. In contrast to the finding of an altered inotropic response to catecholamines, there was no age-associated decrement of these chemicals in shortening contraction duration.

These results suggest that the prolonged duration of contraction of old myocardium may be a compensatory mechanism for the decrease in intrinsic contractile ability. Study of the electrical and mechanical refractory periods of young and old heart muscle demonstrated no age differences in electrical refractory period but a significant prolongation of the mechanical refractory period with age, suggesting that the prolongation of the duration of contraction is due to alterations in the cardiac relaxing system.

2. Osteoporosis is a major problem for the elderly. A Cameron bone mineral scanner utilizing photon absorptiometry has been utilized to assess bone marrow content in participants in the Baltimore Longitudinal Study. Bone mineral content can be estimated with this instrument within an error of .02-.04 grams of calcium per cm of cortical bone radius. Cross-sectional analysis of the data indicated a statistically significant negative correlation between bone marrow content and age. These data will provide a base line for longitudinal assessment to examine the possibility that osteoporosis prone subjects can be identified prior to clinical appearance of metabolic bone disease.

NICHD Annual Report
July 1, 1973 through June 30, 1974
Developmental Immunology Branch

SUMMARY

The immunogenicity and protective effect of the capsular polysacccharides of *Haemophilus influenzae* type b and *Neiserria meningitidis* Group C is being evaluated in Mecklenburg County, North Carolina. The protocol for the preparation of the vaccine and organization of the vaccine trial was constructed according the the recommendations of an ad hoc committee that met with the Scientific Director and Chief of the Developmental Immunology Branch of the NICHD and the Vaccine Advisory Board of the NIAID and was approved by the Deputy Director and Medical Board of the NIH and the Medical Research Committee and Medical Society of Charlotte Memorial Hospital and Mecklenburg County, N.C. A number coded vial of either *H. influenzae* type b or meningococcal Group C polysaccharide will be offered to all infants appearing for their routine immunization program at the offices of private pediatricians, general practioners, the Mecklenburg County Health Stations and the out-patient clinic at Charlotte memorial Hospital. Based upon the age-related attack rate and the number of births in Mecklenburg County annually, we have predicted that, with an 80% participation of the patients, we should be able to statistically verify the vaccine's potential in 2 to 3 years. In addition, we are continuing to study the effect of neonatal stool carriage of cross-reacting *E. coli* upon the acquisition of "natural" *H. influenzae* type b antibodies. Our results indicate that carriers have a more rapid development and higher levels than their age-matched controls. In addition, the infants, previously studied for their immune response to immunization with the *H. influenzae* type b polysaccharide at 2 months have been studied for 2 to 3 years. Their health has been normal and their serum antibodies are higher than their age-matched controls indicating that the immunization-induced antibody response should be long-lived.

The effect of deliberate colonization of newborn rabbits and primates and adult primates and humans with cross-reacting bacteria including *Escherichia coli* strains Easter and "89" (075:Kf147:H5) and *Bacillus pumilis* strain Sh-18 for their heteroimmunognicity to *Haemophilus influenzae* type b is under study. All three organisms successfully colonize the gastrointestinal tract of newborn rabbits and primates for a variable but finite time, lasting from 2 weeks to 6 months. In newborn rabbits a definite and specific immune response was measured in the fed rabbits by examining their in vitro cellular reactivity and their serum antibody and cellular response to sublethal challenge to *H. influenzae* type b organisms at 8 weeks of age as compared to controls. Newborn primates were successfully colonized for 3 to 6 months with the 2 *E. coli* strains. However, controls as well as the fed animals were heavily colonized with nasopharyngeal and stool cross-reacting organisms in addition the fed organisms. All animals reacted with the rapid acquisition of *H. influenzae* type b antibodies almost immediately after their maternal level had declined. The animals did not have any adverse reactions to doses as high as 10^{11}fed at birth. Adult primates required doses of $10^{11}$for successful colonization. The fed and colonized adult primates responded with a 2 to 3 fold rise in serum anti type b antibodies. Six adult humans have been successfully colonized with 10^8 *E. coli* strain Easter. The first four responded with a 2 to 3 fold increase in serum *H. influenzae* type b capsular polysaccharide antibodies. The biological properties of these *E. coli* induced antibodies are under investigation.

Based upon our research program into the study of the immunogenicity of cross-reacting bacteria fed to newborns, we have studied the potential toxicity of these organisms to newborns. In a collaborative study, we have examined *E. coli* isolates from the CSF of 100 neonates for cross reacting antigens to *H. influenzae* type b, meningococcal Groups A,B,C, and pneumococcal types I and III. As controls, we have examined blood and stool isolates from septic and normal newborns and from adults. No *E. coli* with the cross reacting antigen to the *H. influenzae* type b polysaccharide has been detected. However, with these antisera we have found that CSF isolates from newborns react with the meningococcal Group B antiserum indicating that these organisms possess the *E. coli* capsular polysaccharide. In contrast, 50% of newborns with sepsis but not meningitis had *E. coli* with the K1 antigen, and approximately 10% of newborn stool and adult blood and stool cultures had this capsule. The CSF K1 isolates were highly virulent for mice as contrasted to the non-K1 CSF isolates and other non-K1 isolates from the blood and stool. Using serotyping for somatic and flagellar antigens, most CSF organisms could be found in the corresponding maternal stool. A protective effect of meningococcal Group B polysaccharide antibody suggests that immunity to neonatal *E. coli* meningitis may be conferred by intrauterine acquired antibodies. The *E. coli* K1 and meningococcal Group B polysaccharides have been shown to be non-immunogenic in humans. Accordingly, a series of derivatives have been prepared with stearic acid and poly-L-tyrosine for enhancing the immunogenicity of this capsular antigen. The finding of a virulence factor permits several questions to be posed regarding the pathogenesis of *E. coli* neonatal meningitis. Such questions as the role of maternal serum and/or colostral antibodies in conferring immunity, the origin of the infecting *E. coli*, and the epidemiology of the normal carriage may be studied. Preliminary studies of the concentration of the capsular polysaccharide of CSF *E. coli* K1 strains as compared to K1 strains from the stools, urine, etc. show no signficant differences indicating that host factors may be the critical ones in determining the invasiveness of K1 organisms in the newborn.

The structural basis for the cross reactions observed between *H. influenzae* type b, meningococcal Group A and Group C and pneumococcal type III capsular polysaccharides and enteric bacteria is under study. The structure of the *Bacillus pumilis* polysaccharide cross reactive with the meningococcal Group A polysaccharide has been proposed as being similiar to the overall structure of the pneumococcus C polysaccharide. Three distinct polymers consisting of N-acetyl-D-mannosamine phosphate, polyglycerol phosphate and mucopeptide comprise the cross reacting structure. The *E. coli* polysaccharide cross reactive with the meningococcal Group C polysaccharide has been determined to be a polysaccharide of sialic acid with an ethoxy group. The polymer is susceptible to neuraminidase digestion and resistant to HCl-catalyzed methanolysis. Further studies utilizing perturbation and equilibrium techniques such as nuclear magentic and proton resonance studies are planned. The reported structure of the H. influenzae type b capsular polysaccharide has been challenged by our findings of ribitol as a major constituent of the polymer. The cross reactive antigens discovered in gram positive bacterial polyribitol phosphate and in the Kf147 antigen of E. coli may contain this component as the cross reactive moiety. Identification of ribitol by several techniques as well as the observed resistance to periodate oxidation suggest that the H. influenzae type b polysaccharide may consist of a polyribitol backbone with ribose side chains of varying length.

The plasmid directed E. coli enterotoxin, the pathogenic mechanism for diarrheal disease caused by the organism in infancy is under study. The isolation of this protein has been partially accomplished by bulk culture, gel filtration, isoelectric focusing and affinity chromatography. Isolation of this protein is sought to study its biologic activity, and to prepare monospecific and potent antiserum for rapid diagnosis using the antiserum agar technique and to devise a radioimmunoassay to study the protective effect of serum and colostral antibodies to disease caused by the enteropathogenic E. coli strains.

The pneumococcal C-polysaccharide consists of three polymeric substances: mucopeptide, N-acetyl-galactosamine phosphate and ribitol phosphate. These polymers possess different specificities and are linked through phosphodiester bonds of muramic acid phosphate. The specificity of the C-reactive protein, a serum component that is increased in concentration in individuals with inflammation and under the pharmological effect of estrogen and progesterone-like drugs, combines with the N-acetyl-galactosamine phosphate of the C polysaccharide. The structure and function of the C polysaccharide are being investigated by the combined use of cell walls prepared from a non-autolytic mutant and autolytic enzymes from the wild type organism. The structure and bioregulation of the C-reactive protein is being started to characterize the two dimensional structure of these biopolymers as well as to define its function.

The primary structure of the streptococcal proteinase elaborated by the Group A streptococcal has the unique property of containing only a single half-cystinyl residue per molecule. The -SH group of the potential cysteinyl residue is masked by an undetermined functional group. The primary structure of this protein is almost completed as a first step in the understanding of its mechanism of action and the identification of the masking group within the protein. 280 of its 305 residues have been assigned a position.

A symposium was initiated and conducted in collaboration with the National Cancer Institute on the subject of Pneumocystis carinii Pneumonitis. Aspects of the pathogenesis, immune mechanisms, epidemiology, diagnosis, treatment and prophylaxsis of this common complication of constitutive and induced immunologic deficiency will appear in publication form for the first time.

Serial No. HD-I-4
1. Developmental Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Identification and characterization of non-pathogenic bacterial antigens cross reactive with the capsular polysaccharides of meningococcus groups A, B and C, and pneumococcal capsular polysaccharides types I and III.

Previous Serial Number: HD-I-4(c)

Principle Investigator: John B. Robbins, M.D.

Other Investigators:
Rachel Schneerson, M.D.
Emil C. Gotschlich, M.D.
Darrell Liu, Ph.D.
Gerald Schiffman, Ph.D.
Frits Orskov, M.D.
Ida Orskov, M.D.

Cooperating Units:
Rockefeller University, New York, N.Y.
Downstate Medical Center, S.U.N.Y., Brooklyn, N.Y.
W.H.O. International Escherichia Reference Centre, Statens Seruminstitut, Copenhagen, Denmark

Man Years:

Total:	12/12
Professional:	12/12
Others:	None

Project Description:

Objectives: "Natural" and protective serum antibodies to the capsular polysaccharide of meningococcus group A have been detected in approximately 70% of children and young adults despite the scarcity of nasopharyngeal isolates and the virtual absence of disease caused by this organism in the United States for the past 25 years. Further, the wide prevalence of anticapsular antibodies to meningococcus group B and C, pneumococcus types I and III, and H. influenzae type b is not readily explained by asymptomatic carriage of the homologous organism. Accordingly, a search was conducted for a non-pathogenic enteric bacteria with similar capsular polysaccharide structures as the capsular polysaccharides (virulence factors) of these organisms causing purulent infections in man. Characterization of the chemical and immunogenic structures of these cross reacting capsular polysaccharides was sought to provide an explanation for the "natural" antibodies to these bacterial pathogens as well as to consider such cross reacting bacteria as possible immunogens or vaccines.

Methods Employed: Approximately 5,000 bacteria from serotypically defined collections from the Center for Disease Control, Atlanta, Georgia; the World Health Organization, International Escherichia Reference Centre, Copenhagen, Denmark; the defined Bacilli from the collection of Dr. Ruth Gordon, Rutgers University, New Brunswick, New Jersey; as well as requests for samples from all defined bacterial and fungal collections as cited in "World Directory of Collections of Cultures of Microorganisms," Editor, Martin Skerman, Directory made possible by the financial support of UNESCO, and from individuals who have published data from defined collections of non-pathogenic bacteria, have been studied. Cross reacting antigens have been identified in these organisms by immunodiffusion with appropriate antisera. Cross-immunogenicity has been analyzed by measuring the antibodies to the capsular polysaccharides of meningococcus A, B, and C, pneumococcus type III, and H. influenzae type b, by quantitative radioimmunoassay. Cross-immunogenicity has also been analyzed by measuring the antibodies induced by intravenous injection of these cross reacting bacteria.

Major Findings: Several naturally occurring B. pumilis, as well as 7 of the 20 strains of the collection of Dr. Ruth Gordon, were found to possess a cross reacting polysacchride antigen to the meningococcus group A polysaccharide. The cross reacting antigen from all these B. pumilis strains was immunologically identical. The chemical structure of this cross reacting antigen is under study. Our preliminary experiments indicate that it is a multi-chain polymer consisting of N-acetyl-mannosamine phosphate, polyglycerol phosphate, and a mucopeptide. The immunodominant antigen responsible for the cross reaction with meningococcus group A was found to be N-acetyl-D-mannosamine phosphate. Immunization with these cross reacting bacteria induced the formation of meningococcus group A polysaccharide antibody. These bacteria are found in many cheese and cheese products as well as the normal stool of young adults. It has not been reported to be a pathogen of man and may be considered for future immunization programs.

Seventeen E. coli, with a cross reacting antigen to the group C meningococcus polysaccharide, have been found. This cross reacting antigen is a homopolymer of sialic acid that is susceptible to neuraminidase and is cleaved by hydrochloric acid catalyzed methanolysis indicating its structural similarity to both the group B and group C meningococcus polysaccharides. Immunization of animals with these cross reacting E. coli raised group C polysaccharide antibodies, which was bactericidal and mouse protective.

Eleven E. coli and several strains of Bacillus cereus, including a defined strain (ATCC No. 10201) were detected with a cross reacting antigen to the pneumococcus type III polysaccharide. Immunization with these Bacilli as well as the cross reacting E. coli, induced specific precipitating and mouse protective antibodies to type III pneumococcus polysaccharide. The homologous reaction between pneumococcus type III antibodies and the pneumococcus polysaccharide, as well as the reaction with this antiserum and the cross reacting antigens from E. coli and B. cereus, are markedly inhibited by the addition of D-glucuronic acid to the reaction mixture. Further, D-glucuronic acid markedly inhibits the mouse protective activity to pneumococcus type III infection in mice induced by E. coli antisera.

Significance to Biomedical Research and the Program of the Institute: Structural analogs of enteric non-pathogenic bacteria that are immunogenic as well as antigenic, have been detected,to the capsular polysaccharides of bacteria causing invasive disease in humans. The structural basis for this cross reaction has been analyzed. In many cases, these enteric organisms may be responsible for the age-related acquisition of "natural" and protective antibodies to these bacteria. Such bacteria may serve to coordinate the program in our H. influenzae type b research directed toward the study of the acquisition of anticapsular antibodies induced by neonatal feeding of cross reacting bacteria.

Proposed Course of Project: The chemical structure of the cross reacting antigen and the ability of the bacteria to colonize the gastrointestinal tract and induce cross-immunity to pneumococcus and meningococcus in newborn animals and adult humans will be studied over the next two years.

Honors and Awards: None

Publications:

1. Robbins, J.B., McCracken, G.H., Gotschlich, E., Orskov, F., Orskov, I., and Hanson, L.: Escherichia coli K1 Capsular Polysaccharide Associated with Neonatal Meningitis. N. Engl. J. Med., in press.

2. Robbins, J.B., Myerowitz, R.L., Whisnant, J.K., Argaman, M., Schneerson,R., Handzel, Z.T., and Gotschlich, E.C.: Enteric Bacteria Cross-Reactive with Neisseria meningitidis Groups A and C, and Diplococcus pneumoniae Types I and III. Infect. Immun. 6: 651-656, 1973.

3. Robbins, J.B., Gotschlich, E.C., Liu, T.Y., Schneerson, R., Handzel, Z.T., Argaman, M., Parke, J.C., and Myerowitz, R.L.: Bacterial Antigens Cross Reactive with the Capsular Polysaccharides of H. influenzae type b, N. meningitidis Groups A and C, and D. pneumoniae types I and III. In Robbins, J.B., Krause, R., and Horton, R.E. (Ed.): Proceedings of a Workshop on New Approaches for Inducing Natural Immunity to Pyogenic Organisms. U.S. DHEW, PHS, NIH, NICHD and NIAID. Wash., D.C., U.S. Govt. Print. Off., in press.

4. Myerowitz, R.L., Gordon, R.E., and Robbins, J.B.: Polysaccharides of the Genus Bacillus Cross-Reactive with the Capsular Polysaccharides of Diplococcus pneumoniae Type III, Haemophilus influenzae Type B, and Neisseria meningitidis Group A. Infect. Immun. 8: 896-900, 1973.

Serial No. HD-I-8
1. Developmental Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Identification of the immune response to *Haemophilus influenzae* type b capsular polysaccharide induced by feeding of bacteria with cross reacting antigens to newborn rabbits and primates and adult primates and humans.

Previous Serial Number: Same

Principle Investigators: Zeev Handzel, M.D.
Rachel Schneerson, M.D.
John Robbins, M.D.

Other Investigators: James C. Parke, Jr., M.D.

Cooperating Units: Charlotte Memorial Hospital, Charlotte, N.C.

Man Years:

Total:	30/12
Professional:	23/12
Others:	7/12

Project Description:

Objectives: Previous work done in this laboratory showed that immune antiserum, induced by repeated intravenous injection of several cross reacting bacteria, possessed the same immunochemical and "protective" properties as *H. influenzae* type b antisera. The objectives of this project are to identif the effect of "natural" immunity or heteroimmunization to *H. influenzae* type induced by deliberate feeding of newborn and adult rabbits and primates, and adult humans with enteric bacteria containing cross reacting antigens. To follow human newborns with "natural" infections identified after four days in their newborn nursery at Charlotte Memorial Hospital with cross reacting bacteria as to their growth and development and acquisition of serum *H. influenzae* type b capsular polysaccharide antibodies.

Methods Employed: Newborn animals, including rabbits and primates, have been fed two *E. coli* strains designated 075:H5 and *B. pumilis* designated SH-18, which contain a newly described cross reacting antigen to the *H. influenzae* type b capsular polysaccharide. Cross reacting *E. coli*, 075:H5, strain Easte, has deliberately been fed to adult volunteers. Stool cultures, as a measure of gastrointestinal colonization with these organisms, was determined by culturing swabs onto *H. influenzae* type b antiserum agar plates. Measurement of serum antibodies to the H. influenzae type b capsular polysaccharides was done by radioimmunoassay. Cells synthesizing anticapsular antibody as well as lymphoid cells reactive to addition of the purified antigen to cell culture, were measured by plaque-forming hemolysis using lymphoid cells and thymidine incorporation, respectively.

Major Findings: Feeding two strains of cross reacting E. coli or B. pumilis (SH-18) to newborn rabbits exerted no deleterious effect upon their growth and development. At 8 weeks of age, the rabbits fed the cross reacting bacteria had slightly elevated levels of primed gastrointestinal and spleen lymph cells and serum type b antibodies than their controls. Injection of a sublethal dose of H. influenzae type b induced a tenfold increase in serum antibodies and primed cells in all the fed animals as compared to the control.. One strain of cross reacting E. coli, strain 89, seemed to be more immunogeni than the other two strains used for feeding.

Colonization of adult primates was dose-related. At doses of 10^9 bacteria, adult primates could not be successfully colonized. At doses of 10^{11}, 4 out of 5 adult animals fed E. coli, strain Easter, showed positive stool cultures for several weeks thereafter. A statistically significant and sustained serum anti type b antibody response was observed in the colonized animals. Colonization of newborn primates was not dose-related. As little as 10^3 or as high as 10^9 bacteria successfully colonized newborn primates. The colonization was variable, lasting for as long as 8 to 12 weeks with strain Easter, and as short as 3 weeks with B. pumilis. In addition to observing the cross reacting bacteria in the stools of the fed animals, cross reacting organisms were observed in the nasopharyngeal and stool cultures of controlled animals throughout the experiment. Many of these cross reacting organisms were E. coli, Staphylococcus aureus and unidentified strains of Bacillus. Feeding of the newborn animals had no deleterious effect upon their growth and development. Controls, as well as fed animals, showed a rapid decline in the level of maternal antibodies up to age 2 to 3 months. Thereafter, there was a sustained rise of antibodies in all groups which was no difference between the fed and the non-fed animals. Two of the newborn primates fed E. coli, strain 89, had a rapid and short-lived burst of serum anti type b antibody.

Four adult volunteers were fed 10^8 E. coli, strain Easter. All showed positive stool cultures varying from 4 to 8 weeks after feeding. All four volunteers had at least a twofold rise in serum anti type b antibody.

Significance to Biomedical Research and the Program of the Institute: These preliminary experiments show that feeding and colonization of the gastrointestinal tract induced no observed toxicity in newborn rabbits and primates, and adult primates and humans. Feeding the organism to adult primates and humans showed a rapid and sustained serum H. influenzae type b capsular polysaccharide antibody response. No definite immunogenic effect could be ascribed to feeding of the cross reacting organisms to newborn primates; however, these animals were contaminated with multiple cross reacting organisms and all groups showed a rapid serum antibody response at age two months. These experiments show that feeding of cross reacting organisms seems to be a harmless and non-toxic procedure and may be capable of inducing serum anti type b antibody response in human infants by neonatal feeding of these organisms. If this method proves useful, it may be a more convenient way for the prevention of purulent meningitis due to *H. influenzae* type b, the leading cause of acquired mental retardation in the United States.

Proposed Course of Project: Various doses of *E. coli* strain combinations will be evaluated for their immunogenic activity in adults and children for the next year. Our immediate objective is to consider the use of this method of immunization for newborn humans.

Honors and Awards: John B. Robbins

Invited Lecturer, William A. Evans, Jr. Memorial Lecture, Children's Hospital of Michigan, May 1974.

Publications:

1. Robbins, J.B., Schneerson, R., Argaman, M. and Handzel, Z.T.: *Haemophilus influenzae* type b: Disease and immunity in humans. *Ann. Intern. Med.* 78: 259-269, 1973.

2. Myerowitz, R.L., Handzel, Z.T., Schneerson, R., and Robbins, J.B.: Induction of *Haemophilus influenzae* type b capsular antibody in neonatal rabbits by gastrointestinal colonization with cross reacting *E. coli*. *Infect. Immun.* 7: 137-140, 1973.

3. Robbins, J.B., Handzel, Z.T., Schneerson, R., Parke, J.C., Argaman, M. and Robbins, J.B.: Heteroimmunization of primates to *H. influenzae* type b capsular polysaccharide (HITB) by gastrointestinal colonization with non-enteropathogenic *E. coli* (075:K147:H5, strains Easter and 89) possessing a cross reacting capsular antigen (CRA) to HITB. *Pediatr. Res.* 8: 914-922, 1973.

4. Aaron, L., Handzel, Z., Argaman, M., Schneerson, R. and Robbins, J.B.: Monospecific serum for typing *Haemophilus influenzae* type b produced by immunization with *Escherichia coli*, strain Easter. *J. Biol. Stand.* 2: 25-32, 1974.

Serial No. HD-I-9
1. Developmental Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies of the *Escherichia coli* isolated from cases of neonatal meningitis.

Previous Serial Number: Same

Principle Investigators:
John B. Robbins, M.D.
Mark S. Schiffer, M.D.
George H. McCracken, Jr., M.D.
Emil C. Gotschlich, M.D.

Other Investigators:
Frits Orskov, M.D.
Ida Orskov, M.D.
Lars Hanson, M.D.

Cooperating Units:
The University of Texas Southwestern Medical School, Dallas, Texas
The International Escherichia Centre (WHO), Copenhagen
The Rockefeller University, New York, N.Y.
University of Goteborg, Goteborg, Sweden

Man Years:

Total:	26/12
Professional:	20/12
Others:	6/12

Project Description:

Objectives: To characterize the heteroimmunogenicity to *H. influenzae* type b as well as the toxicity of cross reacting bacteria, including *E. coli* and *B. pumilis*, fed to newborns. To determine whether the antigenic cross reactions with the capsular polysaccharide of *H. influenzae* type b could confer invasive properties to the cross reacting bacteria fed to the newborns, a systematic search of *E. coli* isolated from the blood and spinal fluid of human infants was undertaken.

Methods Employed: In collaboration with Dr. George McCracken, Director of The Cooperative Neonatal Meningitis Study Group, other institutions showing interest in neonatal infections, in addition to the 19 participating hospital groups, were requested to send organisms isolated from the blood and/or spina fluid of newborn infants. In addition, the reference collections of the Center for Disease Control and the World Health Organization were studied for cross reacting antigens to H. influenzae type b, meningococcus groups A, B and C, and pneumococcus types I and III. The virulence of the E. coli isolated from the blood and CSF of normal humans and, as controls, E. coli isolated from adult blood cultures, enteropathogenic strains, and normal infant and adult stool cultures were studied for their cross reacting antigens for the appropriate immunodiffusion techniques and for their virulence by the intraperitoneal mucin-enhanced mouse test. Serotyping for H, O and K antigens using the reference reagents at the Escherichia Reference Centre, WHO.

Major Findings: To date, 86 of 100 CSF E. coli isolates from human infants have been shown to contain the K1 capsular polysaccharide. Among these 86 K1 isolates, there were 7 different somatic and 5 different flagella antigens. Sixteen out of 45 neonatal blood E. coli had the K1 capsular polysaccharide with a similar distribution of O and H antigens as those observed from the CSF. In contrast, only 12% of adult blood, urine and stool cultures, and 15% of human infant stool cultures showed E. coli with this distinctive capsular polysaccharide. The median lethal dose for the CSF K1 strains range from 100 to 1,000 organisms. In contrast, non-K1 strains from the CSF or other sources range from 10,000 to greater than 500,000 organisms. A highly protective effect of purified anti K1 capsular antibody was observed in this mouse model, indicating that immunity to invasive disease in the newborn may be conferred by anticapsular antibodies similar to that observed in infants and adults with respect to H. influenzae type b, meningococcus and pneumococcal disease.

Despite antimicrobial chemotherapy, the combined mortality and morbidity in the newborn for neonatal E. coli meningitis is significant. Approximately 1 in every 2,000 to 3,000 live births results in this infectious disease. Using the technique of counter immunoelectrophoresis, a rapid diagnostic test as well as a quantitative estimate of the capsular antigen provides a prognostic tool for evaluating survival without morbidity due to this disease. Using the antiserum agar technique, it has been possible to identify K1-bearing strains from the affected infant and his immediate environment. With the additional tool of serotyping for other antigenic markers, it can be verified that most E. coli isolated from neonatal meningitis originates from the maternal stool. It is, thus, possible to postulate that susceptible infants may be defined as those born without serum anti K1 antibodies and whose mothers are heavily colonized with this organism.

Significance to Biomedical Research and the Program of the Institute: Despite an intensive study of acquired and constitutive immunologic mechanisms, there remains no satisfactory explanation for the occurrence of neonatal sepsis. Sepsis due to gram negative organisms, of which E. coli is the most common, remains a serious source of morbidity and mortality in newborn humans. The finding of a virulence factor permits questions to be posed which can utilize the existing technology as to the pathogenesis, epidemiology, and mechanisms of immunity to diseases caused by this bacteria.

Proposed Course of Project: A contractual mechanism for the study of this organism in four leading university institutions is under design. The program should last approximately three to four years.

Honors and Awards: None

Publications:

Robbins, J.B., McCracken, G.H., Gotschlich, E., Orskov, F., Orskov, I., and Hanson, L.: *Escherichia coli* K1 capsular polysaccharide associated with neonatal meningitis. *N. Engl. J. Med.*, in press.

Serial No. HD-I-10
1. Developmental Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Isolation, characterization and preparation of diagnostic and therapeutic antisera to the heat labile toxin of Escherichia coli responsible for enteropathogenic diarrheal disease in infancy.

Principle Investigator: Zippora Dafni, Ph.D.

Other Investigators: John B. Robbins, M.D.

Man Years:

Total:	26/12
Professional:	14/12
Others:	12/12

Project Description:

Objectives: Isolation, characterization of the physical-chemical, and biologic properties of the heat labile toxin of E. coli. Evaluation of the heat labile toxins immunogenicity and analysis of antisera produced to this antigen as to its diagnostic and therapeutic effectiveness.

Severe diarrheal diseases in infants, especially in nurseries and child care centers, is often due to enteropathogenic E. coli. The pathogenesis of this diarrheal disease is due to the plasmid-directed synthesis by some E. coli of a heat labile exotoxin similar to its biologic effect upon the gastrointestinal tract as the Vibrio Cholera toxin. It is the objective of this study to isolate and characterize the biologic activity of this bacterial protein and to induce high titered antisera to it. The use of this antisera in antiserum agar technique will be evaluated as to its effectiveness for the rapid diagnosis of E. coli induced diarrhea in infants, to use this purified protein as an antigen for study of immunologic mechanisms, as well as the possibility of using this antiserum for therapeutic purposes.

Methods Employed: Macromolecular-free culture fluid has been successfully used to cultivate a specific strain of toxin-producing enteropathogenic E. coli. The culture fluid has been centrifuged and sterilized by sterile filtration. The toxin-containing fluid has been rapidly concentrated by ultrafiltration and then passed through gel filtration and isoelectric focusing procedures to achieve a high specific activity product. The protein has been shown to have a molecular weight in the range of 100 to 300,000. In addition, utilizing its cross reactivity with cholera toxin, purification has been achieved by passage of the partially purified E. coli toxin to an insoluble immunoabsorbent prepared with cholera toxin antiserum.

Major Findings: Partially purified *E. coli* toxin has been shown to have an isoelectric point approximately 4.3. Antiserum has been produced by an injection of the partially purified product. However, its potency is too low yet to assess its usefulness for rapidly diagnosing the presence of a toxin-producing *E. coli* using the antiserum agar technique. The antiserum has been shown to neutralize the toxic effect of the protein. Therefore, it has been proposed that therapeutic antiserum might be prepared by directly immunizing the mammary tissue of a cow producing so-called antitoxic milk.

Significance to Biomedical Research and the Program of the Institute: *E. coli* diarrheal diseases are an important source of morbidity and mortality in humans. In addition, the ever expanding use of nursery facilities for newborns and young children make epidemic disease due to this organism more likely. Isolation of the toxin with production of antisera should permit a more accurate evaluation of its biological activity upon the gastrointestinal tract, production of antiserum which might facilitate the rapid diagnosis of a toxin-producing strain, as well as considering modes of immunization or producing therapeutic antitoxic milk.

Proposed Course of Project: Attempts at purification to homogeneity will continue over the next year. Continual evaluation of the purified toxin as an antigen is also under way.

Honors and Awards: None

Publications: None

Serial No. HD-I-11

1. Developmental Immunology Branch
2. Section on Biochemistry of Microbial Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on Haemophilus influenzae type b polysaccharides.

Previous Serial Number: None

Principle Investigator: Darrell T. Liu, Ph.D.
John B. Robbins, M.D.

Other Investigators: Rachel Schneerson, M.D.

Man Years:

Total:	12/12
Professional:	10/12
Others:	2/12

Project Description:

Objectives: This study is directed toward the chemical characterization of the H. influenzae type b polysaccharide, and to elucidate the nature of other bacterial polysaccharides that are cross reactive with the H. influenzae type b polysaccharide.

Methods Employed: H. influenzae type b polysaccharides were prepared by cetavlon precipitation and phenol extraction and further purified by chromatography on Sephadex G-100. The chemical constituents of the polysaccharides were characterized by gas-liquid chromatography, ion exchange chromatography or amino acid analyzer and sugar analyzer.

Major Findings: Polyribitol-phosphate has been described as a component of "envelope" structures among some common bacteria. Three D. pneumoniae types 15, 29 and 34 contain ribitol phosphate as a component of their capsular polysaccharide and a capsular antigen of E. coli 075:Kf147:H5 was also shown to contain ribitol. All of these bacteria cross react with HITB antiserum. Thus, it seems that this polyol-phosphate is the component responsible for cross reactivity with HITB capsular polysaccharide.

Significance to Biomedical Research and the Program of the Institute: The characterization of chemical and physical structure of the virulence factor of H. influenzae type b, the leading cause of bacterial meningitis, is important because this material is being tested as a vaccine and because the invasive properties of this pathogen are related to it.

Proposed Course of Project: The complete structure of the purified *Haemophilus influenzae* type b polysaccharide will be elucidated by the combined use of both chemical and physical techniques. The biosynthesis of this capsular polysaccharide will also be investigated, especially with regard to the nature of the polymerase involved.

Honors and Awards: None

Publications:

> Argaman, M., Liu, T.Y., and Robbins, J.B.: Polyribitol phosphate: An antigen of four gram positive bacteria cross reactive with the capsular polysaccharide of *Haemophilus influenzae* type b. *J.Immunol.* 112: 649-655, 1974.

Serial No. HD-I-12
1. Developmental Immunology Branch
2. Section on Biochemistry of
Microbial Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on meningococcal polysaccharide: Chemical characterization of the cross-reacting antigens from *E. coli*.

Previous Serial Number: None

Principle Investigators: Darrell T. Liu, Ph.D.
John B. Robbins, M.D.

Other Investigator: Emil C. Gotschlich, M.D.

Man Years:

Total:	12/12
Professional:	10/12
Others:	2/12

Project Description:

Objectives: To study the chemical and antigenic structure of the *E. coli* K capsular polysaccharide antigen cross-reactive with the meningococcal Group C polysaccharide.

Methods Employed: Bacteria are grown in defined media and the polysaccharide antigens were prepared by precipitation with cetavlon followed by deproteinization. Purified polysaccharides were characterized by ion-exchange chromatography to identify amino sugars and neutral sugars. The product was also subjected to nucleomagnetic resonance spectroscopic analysis.

Major Findings: I. An *E. coli* antigen cross reactive with the Group C meningococcal polysaccharide. Three *E. coli* with a cross-reacting antigen to the meningococcal Group C polysaccharide have been found. They have been denoted BOS 12, MT 97 and MT 411. We have not yet identified the morphologic characteristics of this cross-reacting antigen. Injection of formaldehyde-treated BOS 12 and MT 97 induces precipitating meningococcal Group C polysaccharide antibodies. The chemical composition of one of these bacterial cross-reacting antigens has been studied. The purified cross-reacting antigen of *E. coli* strain BOS 12 is a pure homopolymer of sialic acid and in this respect it resembles both the Groups B and C meningococcal polysaccharides The linkages of the sialic acid residues in the BOS 12 and the Group B polysaccharides are most likely identical since both are completely hydrolyzed by neuraminidase. This enzyme is specific for the alpha-glycosidic bond. The meningococcal Group C polysaccharide is resistant to enzymic cleavage and most likely consists of sialic acid residues linked by a beta glycosidic bond. However, unlike the meningococcal Group B polysaccharide, both the Group C and BOS 12 polysaccharides are resistant to cleavage by HCl catalyzed methanolysis. Thus, BOS 12 resembles the meningococcal Group B polysaccharide with respect to its susceptibility to neuraminidase and resembles the meningococcal Group C polysaccharide with its susceptibility to HCl-catalyzed methanolysis. Serologically, BOS 12 will only precipitate with meningococcal Group C antiserum. Rabbit anti-BOS 12 serum will not precipitate with the Group B polysaccharide.

Further studies on the sialic acid polymer isolated from E. coli strain BOS-12 by NMR spectroscopy revealed that it contains an ethoxy group (CH_3-CH_2-) attached to the polymer. The presence of CH_3-CH_2- group in this polysaccharide could conceivably change its conformation and allow it to cross-react with Group C meningococcal antisera and prevent it from reacting with the Group B meningococcal antisera. Elucidation of the detail three dimensional structures of these polysaccharides by means of NMR or light scattering spectroscopy will no doubt contribute to the understanding of the basic principle underlining their immunological properties. Such studies have been initiated.

II. Preparation of polysialic acid similar to the B polysaccharide from E. coli. A polymer of sialic acid was isolated from the cell wall of E. coli K235 by the procedure utilizing cetavlon for extraction. The polymer prepared in this manner is similar in composition but different in physical properties from colominic acid prepared from this organism by Goebel and his associates; the cetavlon product is larger in molecular size. Chemical analysis and enzymic hydrolysis with neuraminidases indicated that the E polysaccharide is very much similar to the B polysaccharide but differs from the C polysaccharide. The E polysaccharide was resistant to the cleavage by methanolic-HCl but it was completely hydrolyzed to the monomeric N-acetyl-neuraminic acid by neuraminidase. Moreover, immunological test revealed that the E polysaccharide reacted with antisera against Group B polysaccharide but failed to react with antisera against Group C polysaccharide. Viscosity measurement suggested that the E polysaccharide could be at least twice as large as the preparation of B polysaccharide currently available for our test. These results suggest that there is a possibility of using E polysaccharide to substitute for the B polysaccharide. Preparation of B polysaccharide for use as vaccine has so far not been successful. Alternatively, partially treated cells of E. coli could also be used to prepare antibody against Group B meningococcus. Cross-reacting antigens produced by unrelated organisms may generate protective activity of antibodies in nature.

Significance to Biomedical Research and the Program of the Institute:
The relationship between the chemical properties of the capsular polysaccharide of meningococcal Group B and C and cross reactive antigens of enteric nonpathogen bacteria is under study. Further characterization of these capsular polysaccharides should provide more information regarding the efficiency of bacterial vaccines.

Proposed Course of Project: The chemical structure of the BOS 12 polysaccharide will be studied in conjunction with the meningococcal B and C polysaccharide by using both chemical and physical methods to elucidate the relationship between structures and functions of these biopolymers. Immunogenicity and chemical characterization should take 2 years.

Honors and Awards: None

Publications:

1. Robbins, J.B., Gotschlich, E.C., Liu, D.T., Handzel, Z., Argaman, M., Schneerson, R., Parke, J.C. and Myerowitz, R.L.: Bacterial antigens cross-reactive with the capsular polysaccharides of *H. influenzae* type b, *N. meningitidis* groups A and C, and *D. pneumonia* types I and III. In Robbins, J.B., Krause, R., and Horton, R.E. (Ed.): *Proceedings of a Workshop on New Approaches for Inducing Natural Immunity to Pyogenic Organisms*. U.S. DHEW, PHS, NIH, NICHD and NIAID. Wash., D.C., U.S. Govt. Print. Off., 1974, in press.

Serial No. HD-I-13
1. Developmental Immunology Branch
2. Section on Biochemistry of Microbial Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Structure and Function of Bacterial Cell Wall: Structure and Immunological Properties of Bacillus pumilis SH-17.

Principle Investigator: Darrell T. Liu, Ph.D.

Other Investigators:
Willie Vann, Ph.D.
John B. Robbins, M.D.

Man Years:

Total:	20/12
Professional:	15/12
Others:	5/12

Project Description:

Objectives: This study is aimed at the characterization of the chemical nature of the Bacillus pumilis SH-17, which is responsible for cross reacting with both group A meningococcus and group A hemolytic streptococcus. The structure of this polysaccharide resembles the pneumococcus C-polysaccharide and thus offers an opportunity for comparative biochemical study on the two biopolymers.

Methods Employed: Polysaccharides were prepared by cetavlon precipitation, phenol extraction followed by purification on Sephadex G-100 column and ion-exchange chromatography on DEAE-cellulose. The components of the polymer were characterized by gas-liquid chromatography, ion-exchange chromatography on amino acid and sugar analyzers.

Major Findings: Chemical-physical studies on group A meningococcal polysaccharide reveal that it is a homopolymer of N-acetyl-mannosamine phosphate with a 1,6-phosphodiester linkage. The molecular weight of the A-polysaccharide suitable for use as vaccine has been determined to be near 150,000. The A-polysaccharide, however, is exquisitely sensitive to autolysis at room temperature. It depolymerizes to smaller, biologically inactive fragments in aqueous solutions. A cross reacting antigen has been prepared by J. B. Robbins from a strain of B. pumilis SH-17, not known to be a human pathogen.

Significance to Biomedical Research and the Program of the Institute: Continued study of the immunogenicity and its relation to the structure of the homologous and heterologous antigens of bacteria causing purulent meningitis is related to our preventive immunization program.

Proposed Course of Project: This polysaccharide, because of its dual antigenicity, will be subjected to immunological studies against both group A meningococcus and group A streptococcus. Its use as vaccine against the two organisms will be tested. The biosynthesis of this polysaccharide will be investigated in conjunction with the pneumococcal C-polysaccharide.

Honors and Awards: None

Publications: None

Serial No. HD-I-14
1. Developmental Immunology Branch
2. Section on Biochemistry of Microbial Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Structure and function of bacterial cell wall: Structure and biosynthesis of pneumococcal cell wall.

Previous Serial Number: None

Principle Investigator: Darrell T. Liu, Ph.D.

Other Investigator: C. J. Lee, Ph.D.

Man Years:

Total:	14/12
Professional:	14/12
Others:	None

Project Description:

Objectives: The pneumococcal C-polysaccharide has the unusual property of combining with and precipitating the C-reactive protein. This protein is not present in the serum of normal humans but appears in the course of many pathological conditions. Work to date has established that the pneumococcal C-polysaccharide consists of three polymeric substances, a polymer of mucopeptide, a polymer of N-acetyl-galactosamine phosphate and a polymer of ribitol phosphate (R36) or glycerol phosphate (CW1). These polymers possess different immunological specificities and are cross-linked through phosphodiester bonds of muramic acid phosphate. Our work also provided information that the specificity of the C-reactive protein is directed toward the polymer of N-acetyl-galactosamine phosphate.

Methods Employed: The structure and function of the pneumococcal polysaccharide are being investigated by the combined use of cell walls prepared from a non-autolytic mutant and the autolytic enzymes prepared from the wild type organism. The autolytic enzymes will be fractionated, purified and used to cleave the cell wall of the non-autolytic organism under controlled fashion to facilitate the study of the total structure of the pneumococcal cell wall polysaccharide.

Major Findings: I. Growth of pneumococci strain R-61 and CW1-1. Two strains of pneumococci were grown in brain-heart medium and a synthetic medium consisting of amino acid mixtures. R-61 is a wild type pneumococci which produces powerful autolytic enzymes. The organism can be lysed by these enzymes. Strain CW1-1 is a mutant derived from R-61 which does not produce autolytic enzymes but can be lysed by enzymes produced from strain R-61. The two strains of pneumococci were successfully grown in brain-heart medium as well as in synthetic medium.

Thomaz has shown that when the pneumococci were grown in synthetic media with ethanolamine replacing choline, the resulting pneumococci is resistant to autolysis and to extremely high doses of antibiotics. We have grown both CW1-1 and R-61 in synthetic media containing ethanolamine. The cell wall prepared from these organisms will be used for the study of their structures. Since a method is available for the analysis of all the components of pneumococcal cell wall including ethanolamine, these cell walls can be used to study the location of both ethanolamine and choline in their structure. Certain tumor cells and immunoglobin combine and precipitate with pneumococcal polysaccharide. It has been suggested that choline play an important role in the binding of the pneumococcal polysaccharide to the immunoglobulin. Our structural study of the pneumococcal cell wall will also be directed toward these studies.

II. Biosynthesis of cell-wall polysaccharide. The biosynthetic enzymes used for the study of the polysaccharide synthesis were prepared from strain CW1-1 which lacks the autolytic enzymes but possesses the synthetic enzymes. The precursors used were: C^{14}-UDP-N-acetyl glucosamine, ^{3}H-N-acetyl-galactosamine and ^{14}C-COP-glycerol. The acceptors were the soluble C-polysaccharide prepared from strain R-61 and the insoluble cell-wall polysaccharide prepared from strain CW1-1. Bacterial membranes were also prepared from both strains of pneumococci and used as acceptors. The mechanism of the elongation of the polysaccharide chains as measured by the incorporation of isotope precursors into the cell-wall membrane and into the soluble and insoluble polysaccharide were studied. There was small but definite incorporation of the isotopes into the membrane fractions. These studies will be continued to elucidate the biosynthesis of the bacterial polysaccharide especially with regard to the mechanism of initiation and termination of the polysaccharide chain synthesis. Cross-linking between the different polymers of polysaccharide will also be investigated by the similar technique using mutant that lacks particular polymers.

Studies on the production of specific antibody from R-61 and CW1-1 types were conducted by immunization of vaccines prepared from each cell-type into the experimental animals: burros and rabbits. After 1 month of initial immunization and 3 weeks of booster immunization, the sera of experimental animals produced specific antibody, as determined by the immunodiffusion technique. R-61 and CW1-1 types contained cross-reacting antigens to each other's specific antiserum. Furthermore, lysed R-61 cells could induce immunoreaction to the antiserum prepared from rabbits immunized with soluble C-polysaccharide.

Significance to Biomedical Research and the Program of the Institute: Precise characterization of the cell-wall biosynthesis of gram-positive bacteria is of interest to our understanding of the heteroimmunization of these substances to the capsular polysaccharide of pyogenic bacteria.

Further, knowledge of the interaction of this substance with the C-reactiv protein should permit characterization of the stimulus for the biosynthesi of this serum protein.

Proposed Course of Project: Isolation of the C-polysaccharide and its ethanolamine derivations will take 1 year. The C-polysaccharide will be utilized for studies of the isolation, characterization and biological studies of the C-reactive protein.

Honors and Awards: None

Publications: None

Serial No. HD-I-15
1. Developmental Immunology Branch
2. Section on Biochemistry of
   Microbial Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on Streptococcal Proteinase

Previous Serial Number: None

Principle Investigator: Darrell T. Liu, Ph.D.

Other Investigators: Joseph Y. Tai, Ph.D.
Joan D. Robbins

Man Years:

Total: 26/12
Professional: 14/12
Others: 12/12

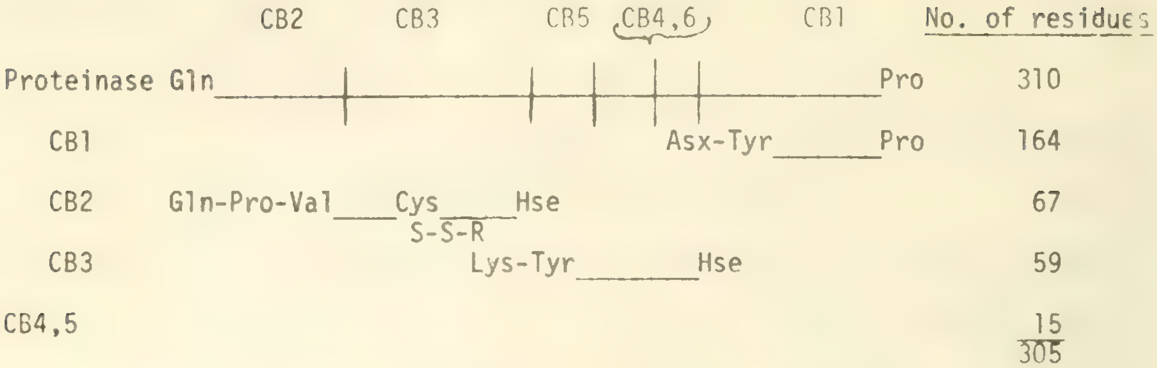
Project Description:

Objectives: Group A streptococci elaborate an extracellular zymogen (M.wt.44,000) which can be transformed into an active proteinase (M.wt.32,000) by proteolysis followed by reduction. The protein has the unique property of containing only a single half-cystinyl residue per molecule. The SH group of the potential cysteinyl residue is masked by an as yet unknown functional group. Studies are in progress on the primary structure of streptococcal proteinase as the first step in the elucidation of the tertiary structure of the enzyme and to help in the understanding of its mechanism of action and the identification of the unknown masking group on the SH group of the enzyme. Mixed disulfides are often utilized by nature for the protection of reactive SH groups in proteins. The identification of such a group, therefore, might have important biological significance.

Methods Employed: Streptococcal proteinase was isolated from the culture of group A streptococci. The proteinase was purified by crystallization followed by ion-exchange chromatography on SP-Sephadex column. The combined use of Edman degradation, chemical cleavage and specific enzyme digestion were employed for the primary structural studies of the proteinase.

Major Findings: The proteinase was first cleaved at the methionyl position with cyanogen bromide. Six peptide fragments were isolated by chromatography on Sephadex G-75 in 2040 acetic acid and sulfaethyl Sephadex chromatography. The alignment of the cyanogen bromide peptides are shown below.

Alignment of Cyanogen Bromide Peptides



Significance to Biomedical Research and the Program of the Institute: Studies on the primary structure and the mechanism of action of this bacterial enzyme will contribute to the understanding of how nature designs a catalyst for specific purposes. Sulfhydryl groups play an important role in the biological function of many proteins. Elucidation of the unique nature of the SH group in the proteinase will help to understand the specific function of some SH group in biologically important proteins and peptides.

Proposed Course of Project: Studies on the primary structure of the proteinase will be completed within one year. The unknown masking group of the SH group will be investigated by the use of gas-liquid chromatography and mass spectroanalysis.

Honors and Awards: None

Publications: None

Serial No. HD-I-16
1. Developmental Immunology Branch
2. Section on Biochemistry of Microbial Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Methods in protein chemistry.

Previous Serial Number: None

Principle Investigator: Darrell T. Liu, Ph.D.

Other Investigator: Richard M. Simpson, Ph.D.

Man Years:

Total:	14/12
Professional:	14/12
Others:	None

Project Description:

Objectives: During the last few years, in connection with our basic research on streptococcal proteinase and cell-wall polysaccharides from pneumococcus and meningococcus, we have developed new procedures for the analyses of tryptophan, cystine plus cysteine and sialic acid. These methods have proven useful not only to our own research projects but also to other researchers in the field.

We have developed physical-chemical criteria for the standardization of vaccines consisting of group A and C meningococcal polysaccharides. We will maintain and update instrumentations and methodology used in both protein and carbohydrate researches.

Methods Employed: Proteins and peptides were subjected to acid hydrolysis utilizing methanesulfonic acid. The hydrolysates were analyzed by ion-chromatography on a Beckman amino acid analyzer equipped with automatic integrator.

Major Findings: Development of a new procedure for the hydrolysis of proteins and peptides. Successful analyses of amino acids in proteins and peptides depends upon (a) the complete cleavage of every peptide bond, (b) prevention of destruction of amino acids during hydrolysis, and (c) a quantitative procedure suitable for the characterization and determination of the amino acids released.

The major method used in the past to hydrolyze protein and peptide samples to amino acids has been that of heating with 6 N HCl. Most proteins, however, contain tryptophan, which is unstable to HCl. It would be a grea advantage to have a method of acid hydrolysis that would yield all the amino acids including tryptophan.

We have chosen sulfonic acid to replace HCl since it is well known that sulfonic acids are non-oxidizing strong acids. In our initial studies, we used 3 N toluenesulfonic acid. The method gave more than 90% recovery of tryptophan from 10 proteins in 22 hour hydrolysates. This method, however suffers from two disadvantages: a) toluenesulfonic acid is a solid which is crystallized from HCl and is therefore difficult to prepare free of HCl, b) the release of valine and isoleucine from some proteins after 22 hours was often incomplete.

We have since overcome these difficulties by using 4 N methanesulfonic acid and a higher temperature for hydrolysis (115° instead of 110°). The results of our experiments show that the yields of tryptophan as well as lysine, histidine and arginine are nearly quantitative. The method should be useful for routine amino acid analyses of proteins and peptides.

II. Development of a simple technique for the quantitative determination of total cysteine and cystine content of proteins. A technique which yields a precise knowledge of the total cysteine and cystine content of proteins will be of considerable value in the elucidation of the relationship between protein structure and function.

At the moment, the quantitative determination of these amino acids is usually made either by analyzing for cystic acid after performic acid oxidation of the protein or for S-carboxymethyl-cysteine after reduction and carboxymethylation of the protein in 8M urea. These methods do not distinguish between the number of free sulphydryl and free disulphide groups in proteins. Furthermore, these methods often contain inherent difficulties in relating the total cysteine and cystine content to the other amino acids in the protein.

Using the technique that we have developed, cysteine and cystine residues in proteins can be independently determined--free sulphydryl groups as S-carboxymethyl cysteine and free disulphide groups as S-sulpho cysteine.

The method is as follows: First, the free sulphydryl groups of the protein are alkylated with iodoacetate using mild denaturating conditions. Then the S-carboxymethylated protein is hydrolyzed (in vacuo), in duplicate, with 4M methane sulphonic acid (containing 0.2%, w/v, 3-(2-amino ethyl)indole) at 115°C.

After hydrolysis, the protein hydrolysate (1.0ml) is adjusted to pH 6.5 by the addition of pyridine (0.3ml) and 4M NaOH (1.0ml), treated with 1.0ml of dithiothreitol solution (6.15mg/ml H_2O) and incubated at 37°C for 1 hour. Solid sodium tetrathionate (60mg) is added and the hydrolysate incubated for a further 16 hours at 25°C, then dried by rotatory evaporation and redissolved in pH 2.2 buffer.

Analysis on the amino acid analyzer yields S-carboxymethyl cysteine corresponding to the amount of free sulphydryl groups in the protein and S-sulphocysteine corresponding to the amount of disulphide groups originall present in the protein.

The results of a typical analysis of papain and ribonuclease A (bovine), indicate quantitative yields of S-carboxymethyl cysteine and S-sulphocysteine. Moreover, the recoveries of tryptophan, histidine and glutamate are also quantitative.

Significance to Biomedical Research and the Program of the Institute: Rapid and complete analysis of small amounts of biologically significant proteins and polysaccharides will be facilitated by this method.

Proposed Course of Project: Investigations will be pursued in order to extend the scope of this technique so that, as well as being able to distinguish between the content of free SH and disulfide groups in proteins, the technique can further distinguish between highly reactive SH such as those involved in enzymatic catalysis and "sluggish" SH groups.

Honors and Awards: None

Publications:

1. Liu, T.Y., and Neuberger, M.: Hydrolysis of Proteins and Peptides wit Methanesulfonic acid. Biochem. Biophys. Res. Commun., in press.

Serial No. HD-I-17
1. Developmental Immunology Branch
2. Section on Biochemistry of Microbial Structures
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Physical properties of meningococcal and Haemophilus influenzae type b polysaccharides.

Previous Serial Number: None

Principle Investigator: Darrell T. Liu, Ph.D.
John B. Robbins, M.D.

Other Investigators: Benjamin Chu, Ph.D.
Emil C. Gotschlich, M.D.
Florence T. Dunn

Cooperating Units: Stony Brook Campus, S.U.N.Y., Stony Brook, N.Y.
Rockefeller University, New York, N.Y.
Brookhaven National Laboratory, Upton, N.Y.

Man Years:

Total:	14/12
Professional:	14/12
Others:	None

Project Description:

Objectives: Composition and chemical properties of the group A, B and C polysaccharides have been investigated by Liu, et al. These polysaccharides were shown to be different chemically, enzymatically, and immunologically. Since biological potencies of the A- and C-polysaccharides as human vaccine have been correlated with molecular size, it should be important as well as interesting to study the physical properties of those polysaccharides. Partial specific volumes, intrinsic viscosities and molecular weights of the group A and C-meningococcal polysaccharides have been made. In collaboration with Dr. B. Chu, we have measured diffusion coefficients of group C meningococcal polysaccharides by means of laser light scattering. Our investigation has convinced us that laser light scattering can be developed into a technique for rapid characterization of polysaccharides, and in combination with other classical techniques, it should give us information on many aspects of equilibrium, such as molecular weight, polydispersity, size and shape of the polysaccharides, as well as transport properties including translational and rotational diffusion coefficients which could not have been obtained easily otherwise. We believe that the technique can be utilized to study aggregation and disaggregation phenomena. Our preliminary studies seem to indicate that the polysaccharides at finite concentrations aggregate. The B-polysaccharide should impose a particularly interesting puzzle. Cross-linking of different groups to form mixed polymers with dual immunogenicity requires a thorough physical knowledge of those polymers in solution. We have also considered the application of laser light scattering to study the kinetics of polysaccharide reactions.

Methods Employed: Polysaccharide samples were subjected to physical characterization utilizing Beckman Model E Ultracentrifuge, Viscometer and laser light scattering. The results obtained have been analyzed by computers to yield weight average molecular weight, intrinsic viscosities and diffusion coefficients of the polysaccharides.

Major Findings: 1. Ultracentrifuge studies on group A meningococcal polysaccharide. The A-polysaccharides used for this study were prepared by the method of Gotschlich, Liu and Artenstein. Two preparations differing widely in size were obtained by repeated fractionation on Agarose gel columns at 4°. Preparation A-1, eluted as a single peak in the void volume of Bio-gel A-1.5. Gotschlich has shown that preparation A-1 is immunogenic in humans and based on its behavior on the Agarose column, a molecular weight of 2×10^6 was suggested for this preparation. Preparation A-2, eluted as a single peak in the retarded position of Bio-gel A-1.5 but was in the void volume of Bio-gel P-150. Gotschlich has demonstrated that preparation A-2 is not immunogenic in humans but it does react with antibodies developed against group A meningococcus. Based on its behavior on the Bio-gel columns, a molecular weight of 150,000 was suggested for this preparation. The molecular weights of A-1 and A-2 were determined by sedimentation equilibrium experiments conducted in a Spinco Model E analytical ultracentrifuge. The high speed equilibrium method of Yphantis was used.

The molecular weights for the A-polysaccharides were calculated by using the values of $\overline{V} = 0.520$ and $\rho = 1.003967$ which were experimentally determined. The average values of the weight averaged molecular weight for the A-1 sample showed no significant variation over the range of concentration studied. Thus, at the concentrations of 0.01, 0.02, and 0.04 per cent (w/v) the $\overline{M}_w$ values were 24,700 25,700 and 24,900. The Z averaged molecular weights $\overline{M}_z$ for the corresponding concentrations were calculated to be 30,800, 27,700 and 25,700. The index of polydispersity as defined by $\frac{\overline{M}_z}{\overline{M}_w}$ were 1.24, 1.08 and 1.03 respectively for the three concentrations of the A-1 sample.

The weight averaged molecular weights $\overline{M}_w$ for the A-2 sample determined at concentrations of 0.01, 0.02 and 0.04 per cent (w/v) were 177,100, 175,100 and 150,100 and that of the Z averaged molecular weights $\overline{M}_z$ calculated for three concentrations were 192,200, 172,500 and 153,700. The polydispersity index $\frac{\overline{M}_z}{\overline{M}_w}$ were 1.09, 1.02 and 0.98 respectively for the three concentrations determined. The conclusions to be drawn from these investigations are as follows:

1. The polysaccharide samples used for our studies are quite homogeneous in size. This is reflected in the polydispersity index $\frac{\overline{M}_z}{\overline{M}_w}$ shown for the A-1

and A-2 samples.

2. At pH 5.6 and at a temperature of 20° the A-polysaccharide did not change its molecular size during the course of a run in the ultracentrifuge over a period of 28 hours.

3. There is nearly a tenfold difference between the molecular weight determined by the ultracentrifugal procedure and the values obtained by the use of the Agarose column. Thus, preparation A-1 was eluted in the void volume of Bio-gel A-1.5 indicating that its molecular weight might be above 2,000,000. By equilibrium ultracentrifugation A-1 was shown to have a weight averaged molecular weight of 167,430 + 17,000. Likewise, preparation A-2 was eluted in the void volume of Bio-gel P-150 indicating that its molecular weight might be larger than 150,000. The weight averaged molecular weight for this sample was found by equilibrium ultracentrifugation to be 25,100 ± 600. This large discrepancy is due firstly to the fact that gel-filtration on Agarose or Bio-gel reflects the effective Stoke's radium rather than the actual molecular weight of the molecules, and secondly, the behavior of the polysaccharide samples on Agarose or Bio-gel would be quite abnormal. In the absence of other reliable physical methods that can be used to determine molecular weight for polysaccharides, the values obtained by the ultracentrifuge studies should be regarded as the ones that represent the more actual molecular weights of the A-polysaccharide.

4. The potency of the A-polysaccharide as vaccine in humans is obviously related to its molecular size. Preparation which has a weight averaged molecular weight above 150,000 exhibits immunogenicity in humans while the one close to 25,000 does not.

Physical properties of group B and C meningococcal polysaccharides. The C-polysaccharides used were prepared by the method of Gotschlich and Liu. Two preparations differing in size were obtained by fractionation on agarose 4B columns. Preparation C-1, eluted as a single peak in the void volume indicated a molecular weight in excess of 2 million. This material is antigenic in humans and serves as a vaccine against group C meningococcal infection. Preparation C-2 eluted as a single peak in the retarded position of agarose 4B and a molecular weight of 500,000 was estimated. Gotschlich has demonstrated that preparation C-2 is not antigenic in humans but it does react with antisera developed against group C meningococcus.

The molecular weights of C-1 and C-2 were determined by sedimentation equilibrium experiments conducted in a Spinco Model E analytical centrifuge. The high speed equilibrium method of Yphantis was used. The average values of the weight average molecular weight for C-1 and C-2 samples determined were 325,000 and 140,000 respectively. The polydispersity index as defined by M_z/M_w were 1.07 for C-1 and 1.05 for C-2 indicating that the polysaccharide samples used for these studies are quite homogeneous with respect to size. These studies revealed that there is a large difference between the molecular weight determined by the ultracentrifugation method and the values obtained by the use of agarose column. This difference must reflect the physical property (size and shape) of these polysaccharides in solution. The abnormality of the behavior of the polysaccharide on agarose column indicates that they are far from the ideal spherical shape. The physical properties of group B meningococcal polysaccharides from several lots of preparations were examined by Sepharose 4B chromatography, viscosity measurements, equilibrium ultracentrifugation and velocity ultracentrifugations. The results of these detailed studies led to the conclusion that group B polysaccharides prepared by a variety of procedures are not suitable as antigens for use in vaccination. The reason for this is that the polysaccharides are in rapid equilibrium among different sizes when in solution. Contrary to the earlier belief that the B polysaccharide is rapidly degraded by neuraminidases and is therefore unsuitable for use as vaccine, evidence has been obtained to suggest that the cause of instability is due most likely to the aggregation and disaggregation phenomenon.

Laser light scattering studies on group C polysaccharide. In collaboration with Dr. B. Chu of the State University of New York at Stony Brook, we have investigated the feasibility of using optical mixing spectroscopy to characterize group C meningococcal polysaccharides and to provide a rapid method for production control of the polysaccharide vaccine based on diffusion coefficient and polydispersity measurements.

The C-polysaccharides were prepared by the method of Gotschlich, et al, using cetavlon to isolate the group-specific meningococcal polysaccharides. Two preparations differing in size were obtained by fractionation on agarose 4B columns. Preparation C-1, eluted as a single peak in the void volume, is antigenic in humans and serves as a vaccine against group C meningococcal infection. Preparation C-2, eluted as a single peak in the retarded position of agarose 4B, has a lower molecular weight. The molecular weights of C-1 and C-2 were determined by sedimentation equilibrium experiments to be about 325,000 and 140,000, respectively.

Light scattering studies indicate that although each fraction is polydispersed, the absence of higher measurements indicates that both fractions have only one dominant maximum in their size-distributions. The polysaccharides are not random and have $\alpha > 0.5$ indicating asymmetry in molecular shape. Measurements of diffusion coefficients by means of optical mixing spectroscopy is relatively fast, is nondestructive and requires only small amounts of samples. Thus, its potential use as a tool for characterization of meningococcal polysaccharides in vaccine production cannot be ignored.

Significance to Biomedical Research and the Program of the Institute: A physical criteria acceptable to Federal Regulatory Agency for production lots of meningococcal groups A and C polysaccharide vaccine have been established. The size and shape of the polysaccharides in solution have been determined These findings may shed light on the biological properties of these biopolymers.

Proposed Course of Project: To extend these physical-chemical studies, a contract mechanism has been proposed to permit collaboration with Dr. Chu's laboratory.

Honors and Awards: None

Publications:

Chen, F.C., Tscharnuter, W., Schmidt, D., Chu, B., and Liu, T.Y.: Measurement of diffusion coefficients of meningococcal polysaccharide by optical mixing spectroscopy. Biopolymers, in press.

NICHD Annual Report
July 1, 1973 through June 30, 1974
Developmental Immunology Branch
Contract and Collaborative Research

Contract Title:	Characterization of the development of the human infant immune and protective response to *Haemophilus influenzae* type b and *Neisseria meningitidis* group C capsular polysaccharides.
Contractor:	Charlotte Memorial Hospital, Charlotte, N.C.
Money Allocated:	$38,000

Objectives: To characterize the immunogenicity of the *H. influenzae* type b and *N. meningitidis* group C capsular polysaccharide injected into young infants and children. To study the duration of the induced serum antibody response and to compare the values obtained in immunized infants with normal controls for several years postimmunization. To determine the effect upon the age-related acquisition of "natural" antibodies to the capsular polysaccharide of *H. influenzae* type b to the presence of cross reacting *E. coli* in the stool of infants at birth.

Methods Employed: All cases of bacterial meningitis within Mecklenburg County, N.C., have been reported to Dr. J.C. Parke for the past three years. In addition, the case records of the three hospitals, Charlotte Memorial, Presbyterian, and Mercy provide all the acute in-patient care for Charlotte as well as the surrounding area of Mecklenburg County. Examination of the case records of these hospitals has been achieved for ten years, and the attack rate, age distribution and racial distribution for bacterial meningitis in Mecklenburg County has been documented. Examinations of the death certificates within these hospitals as well as for the public health records for the county are identical, indicating that our case reporting is accurate. Diagnosis of the etiology of these cases of bacterial meningitis is accomplished by primary bacteriologic isolation as well as newest serologic techniques for grouping the bacteria and for studying the presence of capsular antigens in the CSF and serum of the patients. Nasopharyngeal and stool carriage of the homologous as well as cross reacting antigens utilizes the antiserum agar technique. Serum anticapsular antibodies are measured by radioimmunoassay. Approval for a protective immunization program for *H. influenzae* type b meningitis, the leading type of bacterial meningitis in Mecklenburg County as well as the United States, was granted by the Medical Boards of the NIH, the Charlotte Memorial Hospital, and the Society of Practitioners of Mecklenburg County. To provide an adequate control, *N. meningitidis* group C capsular polysaccharide will be offered to the infants as an additional bacterial capsular polysaccharide vaccine. The vials will be number-coded so that a patient will receive either *H. influenzae* type b or *N. meningitidis* group C capsular polysaccharide.

Major Findings: A total of 69 infants, age 2 to 3 months, have been injected with the capsular polysaccharide of H. influenzae type b. Transient rises have been observed in approximately 90% of these subjects from 1 to 3 months following immunization. At 12 and 23 months, their serum anti type b antibodies were statistically significantly higher than their age-matched non-immunized controls. Of 4,000 infants cultured by the antiserum agar technique at age 4 days before their discharge from the newborn clinic of Charlotte Memorial Hospital, 36 have had cross reacting E. coli detected in their stools. The organism persisted for variable periods of time in approximately one half of the infants. Their serum anti type b antibodies are under study. To date, our impression is that their serum anti type b antibodies are higher than that of their age-matched controls and that all of the infants showing detectable cross reacting E. coli in the stool had levels of antibodies greater than .08 ug/Ab per ml in contrast to approximately 50% of non-carriers which had no detectable antibody from the ages of 4 to 15 months.

Significance to Biomedical Research and the Program of the Institute: Preventive immunization to H. influenzae type b, the leading cause of bacterial meningitis in the United States and throughout the world, will significantly reduce the number of cases of acquired mental retardation. In addition, the mortality and morbidity of this and other forms of bacterial meningitis constitute a significant source of mortality and morbidity in the U.S.A. Characterization of the human immune response to the protective antigens of these bacteria is the subject of this study.

Proposed Course of Project: It is planned that the immunization program will continue for two years and that the results will be followed for three years to determine the protective effect of this course of immunization.

Honors and Awards: John B. Robbins

Invited Lecturer, William A. Evans, Jr., Memorial Lecture, Children's Hospital of Michigan, May 1974.

Publications:

Robbins, J.B., Parke, J.C., Schneerson, R., and Whisnant, J.K.: Quantitative measurement of "natural" and immunization-induced Haemophilus influenzae type b capsular polysaccharide antibodies. Pediat. Res. 7: 103-110, 1973.

NICHD Annual Report
July 1, 1973 through June 30, 1974
Social and Behavioral Sciences Branch

Summary of Research Activities

The research underway this year has been concerned with several interrelated problems, all dealing with early cognitive, motivational and social development. A book has been completed on the differentiation of the early environment. In this monograph, we have presented findings on the relationship between specific dimensions of experience and development during early infancy. The significance of these findings for theories of early development and their implications for intervention programs have been elaborated.

In progress are several investigations which build on the findings and theoretical insights of the previous study on early environmental influences. With a sample from a different socioeconomic background we have attempted to replicate the study on the differentiation of the early environment. In another investigation with this sample we have broadened our focus from the mother-infant dyad to the triad of father-mother-infant. Work is also continuing on the measurement of early infant temperament in an effort to gain greater understanding of the active role of the infant in eliciting stimulation, and how his characteristics mediate the influence of experiences. In addition, two methodological studies are being initiated this year, one concerned with the development of attachment, the other with the development of competence motivation. Another study still in the planning stage, which will be started in the latter part of this year is concerned with parental interaction with premature infants.

Too often conclusions based on a single study are generalized without any attempt at replication with samples that might differ in level and patterning of stimulation. In a replication of the study of early environmental influences we have chosen a sample of infants and mothers from economically more advantaged backgrounds than those in the previous sample. The major purpose is to determine whether the theoretically important findings from the previous study have broader significance. For example, we found that two environmental variables, the mother's contingent responsiveness to the infant's distress and the amount of kinesthetic-vestibular stimulation had strong and pervasive relations with the development of motor, cognitive, and cognitive-motivational functions at six months. If there should be differences in mean levels of stimulation in this new sample, a basic question is whether these variables show the same relationships with measures of infant development.

Research on early development has been concerned almost exclusively with the influence of the mother. The role of the father has been largely ignored. A study recently completed has been concerned with both the father's direct influence on his young infant and his indirect influence as mediated through the mother. Early analyses of the data indicate that the father's expressed attitudes influence the mother-son relationship, but have no effect on the mother-daughter relationship. One area of the mother-son relationship that seems to be especially vulnerable to paternal influence is her handling of feeding. Conflict in the marital relationship is also reflected in disruptive patterns of feeding male infants. These findings emphasize the importance in studying the early environment of considering the interaction of both parents with the infant and with each other.

We are coming to significant reformulations of the problems of early experiences. In addition to looking at the environment in more differentiated ways, we are beginning to take into account the child's role in determining his effective environment. In the study of reciprocal interactions between mother and infant, the central problem is concerned with the effects of the infant's temperamental characteristics on the mother-infant adaptation. Most research on early environmental influences has conceptualized the problem from a unidirectional perspective. In this study we are conceptualizing the environment and the infant as an interactive system. We are attempting to determine how the infant's sensitivities and early characteristics mediate stimulation and how the infant as an active organism elicits responses from his mother. We have studied couples during the last trimester of pregnancy, we have obtained measures of the infant's neonatal characteristics, and then have observed closely the mother-infant interaction at 4 weeks and again at 6 months. The major analyses have not been completed, but several sub-problems have been investigated with these data.

One set of analyses was concerned with relations between several prenatal and perinatal variables and the mother's self-image at one month postpartum. Women who felt that they had received adequate social support during pregnancy, who also evaluated their labor and delivery experiences favorably and who received lower doses of anesthesia tended to have positive self-images. They expressed confidence in their ability to function effectively in the maternal role, in their capacity to relate maturely to others and in their feelings of self worth.

In other analyses we found significant relationships between the analgesia and anesthesia administered during labor and delivery and some aspects of infant temperament at 3 days. The relationships, however, are not simple. The woman's adaptation to pregnancy is related to both delivery medication and neonatal behaviors. In turn the use of local-regional anesthetics is related to the baby's motor immaturity, irritability and diminished attentiveness; analgesia dosage is related only to motor maturity. These findings suggest that there is an interaction between prenatal and perinatal influences and the behavior of neonates.

In a previous study, we have found significant relationships between cognitive-motivational characteristics at 6 months and intellectual development at 3½ years. Measures of the 6 month infant's motivation to assimilate and to master his environment were found to be more highly related to IQ at 3½ years than were measures of his intellectual status at 6 months. Although there has been a great deal of theoretical discussion of the importance of competence motivation, the infant's motivation to master and to have an impact on his environment, there has been very little research. One of the major roadblocks in studying this problem is the lack of techniques for assessing these characteristics. It is especially difficult to measure competence motivation early in infancy. In a previous study, in collaboration with several research centers, we have worked on the development of measures of persistence, i.e., the duration and intensity of involvement with toys, for 6, 12, 24, and 36 month old infants. In the current study we have conceptualized competence motivation more tightly and have elaborated on the measures from the previous study and developed new measures. After pretesting we have arrived at a series of ten tasks. Codes have been developed to record interest in producing effects (obtaining feedback from the environment) and the persistence and intensity of the infant's attempts to solve problems. We are also observing the infant's behavior in a free play setting and have developed a set of comparable codes of the infant's behavior. We plan to use these methods in studying approximately 40 infants at one year of age. For many of these infants we will have environmental measures at 6 months, thus enabling us to look at the environmental factors which facilitate and impede the development of competence motivation.

Work has continued on the refinement of methods for studying the development of attachment, the distinctive emotional tie between mother and infant during the first year of life. One of the problems in this area is that investigators have used different procedures for measuring attachment, and we do not know whether there is any degree of commonality among these different measures. In this study we are investigating the relationship between several indices of attachment: differential response of the infant to mother and to stranger; the degree of protest shown by the infant on separation from his mother and the infant's greeting behavior when the mother returns after separation. Another methodological issue has to do with the generalizability of these behaviors from one setting to another. Therefore, we are making comparisons of the child's behavior in the home with his behavior in similar situations in the laboratory. Observations are also being made of attachment behaviors in the home in situations similar to our experimental situations. During the past year we have pre-tested a variety of observational methods as well as categories for coding these behaviors. Collection of data will be started in this fiscal year.

Although fear of strangers during the second half of the first year has been described in many child development textbooks as an aspect of normal development, we have recently come to question the universality of this behavior. Analyses of data collected in an earlier study indicate that the infant's reactions to unfamiliar adults vary considerably depending on their age, the proximity of the mother and the specific behavior of the stranger. Another aspect of this research was concerned with the factors associated with individual differences in infant's sensitivity to strangers. Few experiential or temperamental variables were found to be associated with the infant's response to strangers, suggesting that it is a situationally determined response. We have also studied infant's reactions to a standardized approach by a stranger and by his mother. The infant's reacted positively to the stranger's initial approach, but some cried when she picked them up. They reacted positively to being held by their mothers, but when their mothers returned to the room after an absence and did not immediately pick them up, some cried. While fear may have been the motivation underlying the infant's cry, we believe it is equally plausible to interpret the crying as a form of communication. To the experimenter the cry might have been a request to be put down, to the mother, a request to be picked up. We believe that these findings raise questions about the interpretation of crying behavior with strangers as primarily indicative of fear.

In addition to the possibility of neurological damage, there are several potentially adverse environmental circumstances associated with prematurity. There has been speculation that the early separation from the mother in the nursery adversely affects her capacity to relate to the infant. Isolation of the premature in the nursery is also associated with significant restriction in sensory input. In an investigation being planned there will be an attempt to differentiate the effects on the infant's cognitive development of neurological damage from the effects of the isolation in the nursery. We shall also attempt to understand how the nursery stay affects the early parent-infant interaction. There have been relatively few observational studies of interaction between parents and premature infants. This study will compare parent-infant interaction in three groups: prematures at risk neurologically, prematures with minimal neurological risk, and a normal full-term control group.

Staff members have been involved in a variety of professional activities, serving on committees and boards of professional societies, and giving consultation to several organizations.

Dr. Juarlyn Gaiter served as a representative of the American Psychological Association to the National Council of Organizations for Children and Youth. She has been a consultant to the Black Child Development Institute and to the National Council for Black Child Development.

Dr. Robert Harmon has been consultant to the Infant Education Center in Washington, D. C.

Dr. Robert Klein organized a symposium, "Comparison of Model Preschool Programs" at the biennial meetings of the International Society for the Study of Behavioral Development. He continues to serve as a consultant on statistical design to the Center for Policy Review of Catholic University.

Dr. George Morgan was a representative of the American Psychological Association to the convention of the National Council on Children and Youth, and has been a consultant to their day care cluster group.

Dr. Frank Pedersen has been a member of the NICHD Clinical Review Committee. At a national conference on improving care for infants of teen-age parents sponsored by the Consortium on Early Childbearing and Childrearing, he was discussion leader in a group concerned with the young father and his children.

He acted as consultant to staff and graduate students, at the University of Delaware in January, on research in early father-child interaction. He was a member of the program committee of the Southeastern Conference of the Society for Research in Child Development.

Dr. Colleen Rand has served on dissertation committees in the Psychology Departments of Syracuse University and of George Washington University.

Dr. Kay Standley is a member of the Board of Convention Affairs of the American Psychological Association. She is also a Consulting Editor for the Journal of Consulting Psychology. She was invited to present an address on "Women Professionals in Architecture" at the annual meetings of the American Institute of Architects in Washington, D. C. She was also invited to participate in a symposium on "Women and Architecture" sponsored by the School of Architecture, Washington University, St. Louis.

Dr. Leon Yarrow has continued to serve on the Governing Council of the Society for Research in Child Development. He is a member of the NICHD Equal Employment Opportunity Advisory Committee. He is serving on the Fertility Regulation Review Panel of the Center for Population Research. He is consulting editor to the Merrill-Palmer Quarterly Journal of Behavior and Development. He has been a member of the program committee of the International Society for the Study of Behavioral Development. Dr. Yarrow was invited to present a lecture on the "Many-sided World of the Infant" at the Institute of Human Development, University of California at Berkeley. At the meetings of the International Society for the Study of Behavioral Development in Ann Arbor, Michigan he was invited to chair a symposium. At the annual meeting of the American Psychological Association in Montreal, Canada, he was a discussant on a symposium concerned with socio-emotional development during early childhood.

There have been several changes in staff during the past year. Drs. Stuart Copans, A. Bradley Soule and David Schachter, Research Associates, completed their two year assignments in the Social and Behavioral Sciences Branch. Ms. Alice Frances, Secretary, resigned from the staff. Our new Research Associate, Dr. Robert Harmon, came on duty July 1973. Dr. Juarlyn Gaiter was appointed a Staff Fellow in August 1973. Ms. Patricia Simon joined the staff as Secretary, September 1973.

Papers Presented at Professional Meetings:

Duchowny, M. S. "Childbearing: The Significance of Pregnancy Orientation and the Childbirth Experience for Maternal Self-Image." Southeastern Conference of the Society for Research in Child Development, Chapel Hill, N.C., March 1974.

Gaiter, J. L. "The Development of Detour Reaching in Infants." Southeastern Conference of the Society for Research in Child Development, Chapel Hill, N.C., March 1974.

Jennings, K. D. "Orientation to the Social vs. Physical Environment: Relationship to Intellectual Abilities of Preschool Children." Southeastern Conference of the Society for Research in Child Development, Chapel Hill, N.C., March 1974.

Klein, R. P. "Multivariate Comparison of Two Model Preschool Programs." International Society for the Study of Behavioral Development, Detroit, Michigan, August 1973.

Morgan, G. A., Levin, B. & Harmon, R. J. "Determinants of Individual Differences in Infants' Reactions to Unfamiliar Adults." Southeastern Conference of the Society for Research in Child Development, Chapel Hill, N.C., March 1974.

Pedersen, F. A. & Schachter, D. M. "Effects of the Father on the Mother-Infant Relationship." Southeastern Conference of the Society for Research in Child Development, Chapel Hill, N.C., March 1974.

Rand, C.S.W. & Jennings, K. D. "The Function of Infant Crying in Stranger Situations." Southeastern Conference of the Society for Research in Child Development, Chapel Hill, N.C. , March 1974.

Standley, K., Soule, A. B. & Copans, S. A. "Childbearing: Relationships Among Pregnancy Orientation, Obstetric Medication, and Neonatal Behaviors." Southeastern Conference of the Society for Research in Child Development, Chapel Hill, N.C., March 1974.

Standley, K. & Soule, A. B. "Women Professionals in Architecture, Law, and Medicine." Symposium on Women in Architecture, School of Architecture, Washington University, St. Louis, Mo., March 1974.

Publications:

Morgan, G. A. and Toomajian, C. A.: A course projection and staffing model used to facilitate a curriculum change. Research in Higher Education. In press.

Soule, A. B., Standley, K., Copans, S. and Davis, M.: Clinical uses of the Brazelton Neonatal Scale. Pediatrics. In press.

Standley, K. and Soule, A. B.: Women in professions: Historic antecedents and current lifestyles. In Hardy, R.E. and Cull, J.G. (eds.): The Young Female: A Study in Career Guidance. Springfield, Ill. In press.

Shereshefsky, P. M. and Yarrow, L. J. (Eds.): Psychological Aspects of a First Pregnancy and Early Postnatal Adaptation. A monograph of the National Institute of Child Health and Human Development. New York, Raven Press, 1973.

Yarrow, L. J. and Goodwin, M. S.: The immediate impact of separation: Reactions of infants to a change in mother figures. In Stone, L.J., Smith, H.T. and Murphy, L.B. (eds.): The Competent Infant: Research and Commentary. New York, Basic Books, 1973.

Yarrow, L. J., Goodwin, M. S., Manheimer, H. and Milowe, I. D.: Infancy experiences and cognitive and personality development at ten years. In Stone, L.J., Smith, H.T. and Murphy, L.B. (eds.): The Competent Infant: Research and Commentary. New York, Basic Books, 1973.

Yarrow, L. J., Klein, R. P., Lomonaco, S. and Morgan, G. A.: Cognitive and motivational development in early childhood. In Friedlander, B.Z., Sterritt, G. M. and Kirk, G. (eds.): The Exceptional Infant, Vol. 3: Assessment and Intervention. New York, Bruner/Mazel. In press.

Yarrow, L. J., Pedersen, F. A. and Rubenstein, J.L.: Mother-infant interaction and development in infancy. In Leiderman, P.H. and Tulkin, S. (eds.): Cultural and Social Influences in Infancy and Early Childhood. Stanford, Stanford University Press. In press.

Yarrow, L.J. and Harmon, R.J.: Maternal deprivation. In Freedman, A.M., Kaplan, H.I. and Sadock, B.J. (eds.): Comprehensive Textbook of Psychiatry. Baltimore, Williams & Wilkins, in press.

Serial No. HD-SB-10
1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Environmental Influences on Cognitive and Personality Development in Infancy

Previous Serial Number: HD-GD-4(c)

Principal Investigators: Leon J. Yarrow, Ph.D., Judith L. Rubenstein, Ph.D., Frank A. Pedersen, Ph.D.

Other Investigators: Myrna Fivel, M.A., Joan Durfee, M.A., Richard Cain, M.Ed.

Cooperating Units: D. C. Department of Public Health, Children's Hospital of the District of Columbia, Group Health Association of Washington, D. C.

Man Years:

Total:	1.10
Professional:	1.00
Other:	.10

Project Description:

Objectives: In this investigation of early environmental influences on cognitive and personality development during the first year of life, one major goal has been to develop an observational instrument to analyze the components of the early environment. A second objective was to study relationships between specific parameters of the environment and specific aspects of development during the first six months.

Methods Employed: The original sample consisted of 70 infants and their caretakers obtained through two well-baby clinics in the District of Columbia and through a private medical care program. Pediatric and neurological examinations were conducted to exclude infants with abnormalities that might be associated with developmental retardation. For the major analyses we selected from the original sample 41 cases in which we felt there was a representative sample of the infant's early experiences. These were cases in which there was a caretaker who had been responsible for the baby for three months or longer, and in which there had been no abrupt environmental changes around the observation period. Data on environmental variables were obtained through time-sampling observations in the home when the infants were five months of age. Measures of infant characteristics were obtained by means of the Bayley Scales of Infant Development and a situational measure of exploratory behavior and preference for novel stimuli.

Major Findings: Previous reports of findings were largely restricted to results of univariate analyses. Some new analyses were performed directed to the question of how environmental variables act in combination with one another. Multivariate techniques were utilized to assess the combined impact of variables. Stepwise multiple regression analyses were computed to see which components of the environment provide the maximum degree of relationship with different aspects of infant functioning. While there were several specific combinations of variables which showed strong relationships, one general pattern emerged involving two environmental variables. The amount of Kinesthetic-Vestibular Stimulation and the Variety of Inanimate Stimulation provided by the mother together showed strong relationships with several infant measures. In order of magnitude, these are: Goal Directedness (R=.68), Psychomotor Developmental Index (R=.61), Mental Developmental Index (R=.53), Object Permanence (R=.52), and Fine Motor (R=.46). These findings emphasize the importance of both the inanimate and social environment.

Kinesthetic-Vestibular Stimulation and Variety of Inanimate Objects also showed significant interactions with several infant measures. For six of the seven significant interactions the infant measure was lowest when both Kinesthetic and Variety of Inanimate Objects were below the median. When only one environmental variable was below the median, an increase in the one had an accelerating effect while a decrease had a depressing effect. When one of these measures was above the median, an increase or decrease in the other had little effect. To illustrate this complex pattern of interactions, we shall describe in detail the results for the Bayley Developmental Index. When both environmental variables were below the median, the mean P.D.I. was 90.4. When Kinesthetic Stimulation was above the median and Variety below the median, the mean P.D.I. was 115. Similarly, when Kinesthetic was below the median and Variety above, the mean P.D.I. was 115.1. When both were above the median, there is no marked increase, the mean P.D.I. is 115.5. These findings emphasize the notion of an optimal level of total stimulation. Perhaps more importantly, our results direct us to a different level of question about the combined impact of several parameters of the environment. Too often we have been willing to accept the fiction of convenience that variables operate in isolation from one another. We need to ask more complex questions that reflect the complexity of the natural environment.

During the past year we have completed a draft of a book reporting both the univariate and multivariate findings in detail. The book discusses their theoretical and methodological implications, and it includes a few case descriptions of selected families. At present the draft is being given editorial criticism by several readers. Publication is expected by midsummer.

Significance to Biomedical Research and the Program of the Institute: This research has significance for theories of early development and has implications for early child care programs. In addition, the techniques developed for home observations will be useful contributions to methodology of studying the early environment.

Proposed Course of Project: This project will be terminated with the publication of the book reporting the major findings.

Honors and Awards: None

Papers Presented at Professional Meetings:

Yarrow, L., Pedersen, F. and Rubenstein, J.: Mother-infant interaction and development in infancy. An invited paper presented at the Wenner-Gren Conference on Cultural and Social Influences in Infancy and Early Childhood in Austria, June, 1973. To be published in Leiderman, P. H. and Tulkin, S. (eds.): Cultural and Social Influences in Infancy and Early Childhood. Stanford, Stanford University Press.

Serial No. HD-SB2 (c)
1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: An Observational Study of Father-Infant Interaction

Previous Serial Number: Same

Principal Investigators: Frank A. Pedersen, Ph. D., David Schachter, M.D.

Cooperating Units: Pediatrics & Obstetrical Service, National Naval Medical Center

Man Years:

Total:	0.35
Professional:	.25
Other:	.10

Project Description:

Objectives: To obtain descriptive data on the early father-infant relationship in natural settings. A methodological component of the study will compare the types of information obtained from interviews of the fathers with data obtained in direct observation of father-infant interaction. The study will also attempt to determine whether there are mediated paternal effects, i.e., whether the father affects the mother's relationship with the infant. A major goal will be to develop a conceptual model for understanding paternal influences as a part of the early environment.

Methods Employed: The sample consists of 39 middle-class fathers and their first-born infants (a sub-group of the sample in Project HD-SB4(c). At age four weeks the father was interviewed regarding the early postnatal period, his current relationship with the infant, and aspects of the husband-wife relationship which may bear on how each parent relates to the baby. At age five weeks an observation was conducted in the home, usually in the early evening, focusing on the father's interaction with the baby. The observational system is an adaptation of the method described in Project HD-SB4(c). Data from that project on the infant and the mother-infant relationship will be utilized in certain analyses.

Major Findings: The initial data analyses have been directed toward the question of whether the father's relationship with his infant and the husband-wife relationship affect the quality of interaction between mother and infant. Correlations were examined between the ratings of fathers derived from the interviews and independent ratings of the mother-infant interaction made after observations in the home. The data indicate that the father has an impact on the mother-infant interaction but his influence appears more clear-cut with male infants. One area of the mother's behavior that is especially sensitive to the father is her appropriateness in managing feeding. We found that there were differences in the mother's behavior which were related to the affect which the father expressed towards his son. The wife of a man who reported more irritability and negative affect towards his infant son was likely to be rated as more inappropriate in her management of feeding. Similarly, when the husband reported greater tension and conflict in the marriage relationship, his wife was more likely to be rated as inept and disruptive in the pacing and timing of her son's feeding. Interestingly, the father's relationship with his daughter appears to have little influence on the mother-daughter unit. These results as well as other findings were presented at the Southeastern Conference of the Society for Research in Child Development. Additional analyses are being pursued to see whether there are certain behavioral characteristics of male infants which may contribute toward the relationships described.

Significance to Biomedical Research and the Program of the Institute: Most of our knowledge of early parental behavior is limited to the mother-infant unit. The apparent assumption that this is a closed system, relatively unaffected by the father, and that early paternal behavior is of no consequence is open to question. The observational data of father-infant interaction will be unique in their own right, and it is hoped that this study will contribute to our understanding of the infant's early environment.

Proposed Course of Project: The initial findings will be prepared for submission to a professional journal and additional analyses will be pursued in the coming year.

Honors and Awards: None

Publications: Pedersen, F. A. & Schachter, D. M. "Effects of the Father on the Mother-Infant Relationship." Southeastern Conference of the Society for Research in Child Development, Chapel Hill, N. C., March 1974.

Serial No. HD-SB4(c)
1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Effects of Infant Temperamental Characteristics on Mother-Infant Adaptation

Previous Serial Number:

Principal Investigator: Leon J. Yarrow, Ph.D.

Other Investigators: Michael Duchowny, M.D., Robert Klein, Ph.D., Kay Standley, Ph.D., Richard Cain, M.Ed., Myrna Fivel, M.A.

Cooperating Units: Pediatrics and Obstetrical Services of National Naval Medical Center; Childbirth Education Association

Man Years:

Total:	5.75
Professional:	5.50
Other:	0.25

Project Description:

Objectives: To study the effects of infant temperamental characteristics on patterns of maternal care and attitudes towards the infant. Within this overall objective there are a number of specific methodological and substantive questions:

1. Is there a basic consistency between infant characteristics assessed in the neonatal period and infant characteristics at four weeks?
2. What are the relationships between mother-infant adaptation at four weeks and certain prenatal variables: the mother's attitudes and expectations regarding the infant; the husband-wife relationship during pregnancy; the woman's physical and psychological adaptation to the pregnancy experience?

3. To what extent do infant temperamental characteristics influence maternal caretaking patterns and mother-infant adaptation? For example, does a discrepancy in activity level between mother and infant lead to disturbed adaptation? Is there a relationship between the infant's visual alertness and amount of visual stimulation provided by the mother?

Methods Employed: The sample consists of 46 parents and their first-born infants, 24 boys and 22 girls. Only normal infants, free from complications during pregnancy and delivery have been included in the study.

There are five phases of data collection:

1. Prenatal interview with prospective parents in which basic demographic data are obtained. Both parents are interviewed about their expectations and feelings about the unborn child, the degree of solidarity between husband and wife and general adaptation to pregnancy.
2. Neonatal assessment of infant in newborn nursery. Infant characteristics such as visual alertness, auditory responsiveness, irritability, consolability are evaluated using the Brazelton Neonatal Assessment Scale.
3. Four week assessment of infant using the Brazelton Scale.
4. Four week time-sampling observation of mother-infant interaction in the home.
5. Interview with the mother at four weeks regarding her feelings towards the infant and her perception of infant characteristics.

Major Findings: Data collection has been completed on 46 cases. The main analyses have not been completed, but several sub-analyses have been carried out on other aspects of this problem.

One set of analyses was concerned with relations between several prenatal and perinatal variables and the mother's self-image at one month postpartum. Women who felt that they had received adequate social support during pregnancy, who also evaluated their labor and delivery experiences favorably and who received lower doses of anesthesia tended to have positive self-images. They expressed confidence in their ability to function effectively in the maternal role, in their capacity to relate maturely to others and in their feelings of self worth.

In other analyses we found significant relationships between the analgesia and anesthesia administered during labor and delivery and some aspects of infant temperament at 3 days. The relationships, however, are not simple. The woman's adaptation to pregnancy is related to both delivery medication and neonatal behaviors. In turn, the use of local-regional anesthetics is related to the baby's motor immaturity, irritability and diminished attentiveness; analgesia dosage is related only to motor maturity. These findings suggest that there is an interaction between prenatal and perinatal influences and the behavior of neonates.

Significance to Biomedical Research and the Program of the Institute: Most research on early development has concentrated on environmental influences on the young infant, conceptualized in essentially unidirectional terms, i.e., the effects of environmental events on infant development. This model is being replaced by an interactional one in which the child is seen as an active agent who influences the behavior of other people towards him. This investigation should begin to give some understanding of reciprocal interactions between infant and mother.

Proposed Course of Project: Analyses of data will be carried out during the coming fiscal year.

Honors and Awards: None

Papers Presented at Professional Meetings:

Duchowny, M. S. "Childbearing: The Significance of Pregnancy Orientation and the Childbirth Experience for Maternal Self-Image." Southeastern Conference of the Society for Research in Child Development, Chapel Hill, N. C., March, 1974.

Standley, K., Soule, A. B. & Copans, S. A. "Childbearing: Relationships Among Pregnancy Orientation, Obstetric Medication, and Neonatal Behaviors." Southeastern Conference of the Society for Research in Child Development, Chapel Hill, N. C., March, 1974.

Serial No. HD-SB5
1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Development of Measures of Social and Motivational Functions in Young Children

Previous Serial Number: Same

Principal Investigators: Leon J. Yarrow, Ph. D., Colleen S.W. Rand, Ph. D., Kay D. Jennings, Ph. D.

Other Investigators: Sarah Friedman, M.A.

Cooperating Units: Institute for Development of Human Resources, University of Florida; Developmental Research Laboratory, Temple University; Syracuse University Children's Center

Man Years:

Total:	2.0
Professional:	2.0
Other:	

Project Description:

Objectives: Although there are many tests of cognitive functioning for young children, there are few standardized instruments for measuring other important aspects of development. In this study our objective has been to develop measures of social and motivational functions, i.e., persistence and trust, for use with children between six months and three years. This has been a collaborative undertaking with the University of Florida, Syracuse University and Temple University.

Methods Employed: Subjects were white middle-class infants tested within a week of their six and twelve month birthdays and within three weeks of their second and third birthdays.

The Bethesda group has concentrated on developing a series of measures for six and twelve month infants.

Major Findings: Measures of trust in strangers and persistence with objects have been developed for all age groups. In Bethesda, 43 infants have been studied with these measures: 32 at 6 months, 35 at 12 months, 15 at 24 months and 11 at 36 months. A report has been prepared describing the administration of these measures. Statistical analyses of the age and sex distributions of scores on tests are presented, and suggestions for further refinement of these measures are discussed.

Significance to Biomedical Research and the Program of the Institute: Measures that have been used to evaluate compensatory educational programs or the effects of day care programs on the development of young children have for the most part been limited to cognitive development. The measures developed in this project will provide means of assessing other important aspects of development that may be influenced by these programs.

Proposed Course of Project: Data collection has been completed and a report has been prepared.

Honors and Awards: None

Papers Presented at Professional Meetings:

Leon J. Yarrow and Colleen S. W. Rand participated in a conference devoted to a discussion of these measures at the annual meeting of the American Psychological Association in Montreal, Canada, in September 1973.

Publications:

Gordon, I.J., Beller, E.K., Lally, J.R., Yarrow, L.J., Moreno, P., Rand, C. & Freiberg, K. Studies in socio-emotional development in infancy. Office of Child Development, November 1973.

Serial No. HD-SB6
1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Consistency and Antecedents of Infant Attachment During the First Year

Previous Serial Number: None

Principal Investigators: Robert P. Klein, Ph. D., Leon J. Yarrow, Ph. D.

Other Investigators: Joan Durfee, M.A., Myrna Fivel, M.A., George Morgan, Ph. D.

Cooperating Units: None

Man Years:

Total:	1.70
Professional:	1.50
Other:	.20

Project Description:

Objectives: The first objective is methodological. The studies that have been done on attachment have used a variety of techniques--interview with the mother, structured situations in the laboratory--and a variety of indices to measure attachment. In this study, we plan to compare data obtained from observations in the home, with data from an interview with the mother and from observations of structured situations in the laboratory. Underlying this methodological question is the conceptual issue of what are the most appropriate behavioral measures of this relationship. In addition to the methodological question we plan to examine the antecedents of the distinctive tie between mother and infant. Approximately half the sample will have been observed at six months. Several of the environmental variables observed at that time have been hypothesized to relate to later attachment, e.g., amount of kinesthetic stimulation and mother's responsiveness to baby's distress.

Methods Employed: The sample will consist of forty mothers and their twelve month-old infants, 20 boys and 20 girls, with half of each sex being first and half later born. About half of the sample will have been seen at six months (Project No. HD-SB7).

Two home observations of two hours duration, approximately a week apart are conducted within two weeks of the baby's first birthday. Observations are made of selected mother and infant behaviors in a number of critical situations which are comparable to measures that have been used in laboratory studies of attachment behavior. The infant's reactions to such events as the mother entering the room, leaving the room, the appearance of the mother when the infant is crying are recorded. The infant's vocalizations, smiles, approach behaviors, cessation of crying are also recorded.

Following completion of the two home observations, the mother and infant are brought to the Social and Behavioral Sciences' playroom for a series of observations.

1. The infant's initial response to the experimenter/interviewer is observed from behind a one-way mirror. The experimenter approaches the infant in a pre-determined, structured but natural manner. The mother is then asked to go through the same approach.
2. The mother is interviewed, using a semi-structured interview schedule regarding selected aspects of her baby's social functioning in relation to herself, the baby's father, other familiar people, and strangers
3. During the interview, the infant is provided with toys and his exploratory behavior and social initiative and responses (vis a vis mother and interviewer) are observed from behind the one-way mirror.
4. At the completion of the interview proper, the mother is asked to leave the room and the infant's reactions to being left with the interviewer and to the mother's return are recorded.

Major Findings: It was hoped that we could begin data collection last spring in order to include in our sample families we had first seen prenatally. After we had observed about ten infants it became clear that the system needed further development. Because there was a gap of several months until the next group of subjects from our six months study we had time to further refine the system. The problems with the system centered around the fact that infant behavior occurring in conjunction with certain critical events (e.g. maternal departures and reentries) are particularly important for studying attachment. A straight time sampling approach would not allow us to link infant behavior to these events. Furthermore the system has to be simple enough to allow for coding behavior "live." During the past year, we have spent a good deal of time observing infants in their homes and modifying the system so that it is useable in the complicated circumstances of naturalistic home observations.

Significance to Biomedical Research and the Program of the Institute: The study of "attachment" is central to understanding the development of affectional relationships. Most of the research on attachment has been carried out in contrived situations of a rather stressful nature. There has been little comparison of reactions of infants in these experimental situations with behavior in natural settings.

Proposed Course of Project: Data collection will be carried on during the next fiscal year.

Honors and Awards: None

Serial No. HD-SB7
1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Early Environmental Influences on Cognitive and Personality Development During Infancy: A Replication with a Middle-Class Sample

Previous Serial Number: HD-SB7

Principal Investigator: Leon J. Yarrow, Ph. D.

Other Investigators: Robert P. Klein, Ph. D., Frank A. Pedersen, Ph. D., Joan Durfee, M.A., Myrna Fivel, M.A., Juarlyn Gaiter, Ph. D., Richard Cain, M.Ed.

Cooperating Units: None

Man Years:

Total: 1.50
Professional: 1.25
Other: .25

Project Description:

Objectives: In a previous study with a sample composed largely of infants and mothers of low socioeconomic status some significant relationships were found between a number of early environmental variables and infant development. These findings had important implications for developmental theory. Because of their theoretical significance, it seemed important to test the generality of these findings to determine whether similar relationships would be found in other environmental contexts with different levels and patterns of stimulation. Therefore, we are replicating the previous study with a middle-class sample.

Methods Employed: The sample consists of 41 infants, 20 boys and 21 girls and their mothers. The infants were first studied as neonates in the reciprocal interaction project (see Project No. HD-SB4).

Data on the environmental variables are being obtained by time-sampling observations in the home using the mother-infant interaction schedule developed in the previous study. Measures of infant characteristics are obtained from the Bayley Scales of Infant Development and through a situational test designed to measure exploratory behavior and preference for novel stimuli.

Major Findings: Data have been collected on 40 subjects.

Significance to Biomedical Research and the Program of the Institute: All too frequently conclusions with significant theoretical and practical implications are based on a single study in limited experimental or natural situations in which the sample is restricted to one cultural group. If we are to make meaningful generalizations about the significance of specific aspects of early experience for development, it is necessary to examine these relationships in a variety of environmemtal contexts, with samples that might differ in level and patterning of stimulation.

Proposed Course of Project: Data analyses will be completed during the next fiscal year.

Honors and Awards: None

Serial No. HD-SB-11
1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Developmental and Situational Factors in Children's Responses to Strangers

Previous Serial Number:

Principal Investigators: Colleen S W. Kard, Ph. D., Kay D. Jennings, Ph. D.

Cooperating Units: None

Man Years:

Total:	0.75
Professional:	.70
Other:	.05

Project Description:

Objectives: The present study has three main objectives: 1) to map out developmental changes in reactions to strangers from 6 months to 3 years; 2) to determine the prevalence of negative reactions at each age level; 3) to determine situational and other factors leading to negative reactions.

Methods Employed: One hundred infants and young children were tested within a week of their 6 and 12 month birthdays and within 3 weeks of their second and third birthdays.

At 6 and 12 months, the stranger approached the infant in a graduated series of steps which ended with the stranger picking up the infant and playing with him. At two years, the stranger tried to engage the child in a series of activities with her, including leaving the room to fetch a new toy. At three years, the stranger also tried to engage the child in a (different) series of activities; for this age group only, the mother was not in the room with the child.

At each age level, the mother repeated several of the stranger's activities with the child in order to enable comparison of the child's reaction to the stranger and his mother when both engaged in the same behavior.

Major Findings: Data analysis for the 6 and 12 month old subjects has been largely completed. In general, 6 month old subjects were more positive to the stranger than were 12 month old subjects. There were no sex differences at either age level. Looking at reactions to the particular behavior of the stranger, infants were generally positive to the stranger when she simply talked to them; they were also positive when she patted their hand. They became more negative when the stranger picked them up and played with them.

The general conclusion reached was that infant crying should be considered a communication, with fear but one of many possible motivating emotions. Infants cried as much to their mothers as to the stranger overall. Infants, however, reacted more positively when the stranger simply talked to them than when she held them, whereas to their mother they reacted more positively to being held than to being talked to from a distance. While fear might have motivated crying to both the mother and stranger, a more plausible interpretation would be that different infant messages were being communicated: e.g., "Put me down!" to the experimenter and "Pick me up" to the mother.

Significance to Biomedical Research and the Program of the Institute: Until recently "fear of strangers" was accepted as a necessary part of normal infant development in the second half year of life. This view has been challenged recently. In the present study, children's reactions to strangers were examined with a more open eye than past studies because it was not assumed that crying in the presence of a stranger automatically indicated fear of the stranger.

Proposed Course of Project: A paper on these findings and conclusions are now being written and will be submitted for publication. The project will be completed by the end of the current fiscal year.

Honors and Awards: None

Papers Presented at Professional Meeting:

Rand, C. & Jennings, K. "The Function of Infant Crying in Stranger Situations." Southeastern Conference of the Society for Research in Child Development, Chapel Hill, N.C., March 1974.

Serial No. HD-SB-12

. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Determinants of Infants' Reactions to Strangers

Previous Serial Number: None

Principal Investigator: George A. Morgan, Ph.D.

Other Investigators: Robert Harmon, M.D., Robert Klein, Ph.D., Juarlyn Gaiter, Ph.D., Joan Durfee, M.A., Bonnie Levin, B.S.

Cooperating Units: None

Man Years:

Total:	.65
Professional:	.40
Other:	.25

Project Description:

Objectives: In the second half of the first year of life many infants avoid contact with and/or protest the approach of an unfamiliar person. This reaction is especially salient because it contrasts so markedly with the typical unreservedly positive response of 2 to 6 month old infants. The broad aim of this work is to identify and sort out the factors which determine the likelihood that babies will react negatively to strangers.

Methods Employed: The original sample consisted of 80 4 to 13 month old infants. These data were collected several years ago at Cornell University. Two types of data were obtained. First, mothers were interviewed at home when the infant was asleep. Questions were asked about the infant's past and present contacts with familiar and unfamiliar people; the infant's temperament; his reactions to separation from mother; and his reactions to strangers. A few days after the interview, the infant was brought to a comfortably furnished room for systematic observation of his reactions to two unfamiliar adults. During the approximately 30 minute session, the strangers interacted with the infant 12 times, using several standardized approach sequences. The infants' facial expressions, vocalizations and postural activity were recorded every three seconds.

In order to attempt to elaborate on the findings from the earlier study, 40 new infants will be studied in conjunction with the attachment and competence projects. The infant's initial reactions to an unfamiliar adult will be observed in each of five sessions between 11½ and 13½ months, three times at home and twice in our laboratory playroom. In two of the sessions, after the stranger has attempted to initiate a playful interaction with the infant, the mother will be asked to perform a similar sequence of behaviors. Information about the infant's exposure to people and reactions to strangers will be gathered by an interview at 12 months and updated a month later.

Major Findings: Most of the data from the Cornell sample have been summarized in several publications and presentations at professional meetings. These data indicate that infants' reactions toward unfamiliar adults vary considerably depending upon a) their age, b) their proximity to the mother when approached, and c) the specific actions of the stranger. There were few negative reactions toward strangers by the 4 and 6 month old infants in any of the several types of approach sequences. Likewise, the 10 and 12 month old infants were predominantly positive (smiling, reaching toward, etc.) or sober when the stranger approached and touched them while they were on their mother's lap and when he moved his head as if playing peek-a-boo. However, most of these 10 and 12 month old babies reacted negatively (frowned, pulled back, cried, etc.) when approached while four feet from mother and when picked up. These findings of marked situational differences are important because until recently research has not dealt systematically with this issue and it has been essentially overlooked in the theoretical discussions of stranger anxiety. Since there has also been concern about the representativeness of standardized laboratory stranger approach sequences, another significant finding was that mothers' interview reports of their infants' usual reactions to strangers were related to the laboratory observations. Longitudinal data from a follow-up questionnaire provided some evidence that by 16 months infants respond less negatively to strangers than at one year.

The interview data indicated that infants who had had little physical interaction with relatively unfamiliar adults at 3 months but who had more contact with strangers in the month prior to observation, tended to react negatively to strangers in the laboratory. However, little support was provided for hypotheses predicting more negative reactions associated with: 1) maternal protectiveness, 2) the infant's cognitive confusion due to similarity of the parents and the stranger, 3) the infant's concern about separation from mother, or 4) the infant's irritability, lack of adaptability, or sensitivity to stimuli. Although these hypotheses seem plausible, a careful look at the literature indicates that only scattered results in support of them have been reported.

Significance to Biomedical Research and the Program of the Institute: Infants' concern about the approach of strangers is important because it may provide us with early clues about the development of his attachment to his mother and the development of his trust of other people.

Proposed Course of Project: During the next fiscal year papers from two previous convention presentations will be submitted for publication. Analyses on the original sample will be concluded with a detailed examination of the behavioral components and situational determinants of infant's reactions to strangers.

Data on the replication sample will be collected during the next fiscal year.

Honors and Awards: None

Publications:

Morgan, G. A. Fear of strangers in human infants. In L. M. Brockman, J. H. Whiteley and J. P. Zubek (Eds.), *Child Development: Selected Readings*. Toronto: McClelland and Stewart Ltd., 1973, 138-148.

Morgan, G. A. and Ricciuti, H. N. The Morgan-Ricciuti method. In E. G. Boyer, A. Simon, and G. R. Karafin (Eds.) *Measures of Maturation: An Anthology of Early Childhood Observation Instruments*. Philadelphia: Research for Better Schools, 1973, 1505-1532.

Serial No. HD-SB-13
1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Measurement of Competence Motivation in One Year Olds

Previous Serial Number: None

Principal Investigators: Leon J. Yarrow, Ph. D., George A. Morgan, Ph. D.

Other Investigators: Robert J. Harmon, M.D., Juarlyn L. Gaiter, Ph. D.,
Kay D. Jennings, Ph. D.

Cooperating Units: None

Man Years:

Total:	2.15
Professional:	2.05
Other	.10

Project Description:

Objectives: Although there has been a great deal of theoretical discussion on the development of motivation to master and to have an impact on the environment, there has been little research. In a previous study we found marked individual differences in cognitive-motivational characteristics in infants at 6 months. We believe that such motivational differences may be affected by the early environment and, in turn, may be a precursor of later intellectual functioning and later feelings of competence. One of the major obstacles to research in this area is the lack of techniques for assessing competence motivation in infancy. In this study we plan to develo and refine methods to measure one year old infants' motivation to solve problems and to have an effect on the environment. We are also interested in the environmental antecedents of competence motivation and shall look at the relationships between the infant's environment at 6 months and his competence motivation at one year.

Methods Employed: The sample will consist of forty mothers and their infant, 20 boys and 20 girls. Some of the babies will be part of a sample first studied prenatally for another project, HD-SB4(c). Most of the subjects will have entered the study at 6 months as a part of the replication study on early environmental influences, project HD-SB7.

Each infant and mother will be seen three times: at 12 and 13 months in our playroom and at 13 months in the home.

During the 12 month playroom session the infant will be observed in free play while the mother is being interviewed. The session will be recorded on videotape. After the session, the competence team will code in considerable detail the baby's activities with each of the objects, e.g., mouthing, putting objects in containers, stacking, fitting two objects together.

During the 13 month playroom session, the infant will be presented with several problems most of which are relatively difficult for infants of this age. There are several types of items. Some require the infant to reach around a barrier to obtain an object. For example, a toy will be placed behind a glass screen. Other problems require use of objects as tools or intermediaries. For example, a toy will be placed out of reach of the infant on a pad which he can reach and pull toward him to obtain it. Several items will provide opportunities to produce visual and/or auditory feedback by the infant's actions. For example, by pushing or pounding a lever he can make balls bounce around inside a plastic cylinder. Both global ratings and detailed behavioral descriptions will be made. The following types of measures will be derived: amount of time spent touching the apparatus, amount spent attempting to solve the problem or produce the planned effect, the number and intensity of such effectance behaviors, the variety of ways in which the infant attempts to solve the problems, and the amount of time required to solve each problem.

During the 13 month home visit, the baby will be given a standardized infant developmental test, the Bayley Scales of Infant Development. Then the mother will be interviewed about several of the topics discussed in the 12 month interview. In addition to the cognitive measures on the Bayley Scales, an object permanence test will be given to measure the infant's level of awareness of the existence of objects when they are out of view.

Major Findings: In a previous study we found that some measures of the infant's capacity and motivation to have an effect on the environment were related to intellectual development at 3½ years (Yarrow, Klein, Lomonaco and Morgan, in press).

For the present study, a set of problems which elicit attempts to produce an effect or attain a goal have been designed and pretested. Methods for observing and recording this problem solving behavior and behavior towards objects during free play have been developed.

Significance to Biomedical Research and the Program of the Institute: In recent years there has been an overemphasis on the measurement of cognitive functions in early childhood, and relatively little attention has been given to motivational functions. We think that competence and effectance motivation in infancy may have important implications for the development of competence and self-esteem in later childhood. Moreover, it is likely that the poor predictability of measures of intellectual development during infancy may be partly related to our failure to consider the role of motivational factors.

Proposed Course of Project: Data collection will be carried on during the next fiscal year.

Honors and Awards: None

Publications:

Yarrow, L. J., Klein, R. P., Lomonaco, S. & Morgan, G. A. Cognitive and motivational development in early childhood. In B.Z. Friedlander, G.M. Sterritt and G. Kirk (Eds.), The exceptional infant, vol. 3: Assessment and intervention. New York: Bruner/Mazel, in press.

Serial No. HD-SB-14
1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Early Parent-Infant Interaction and Cognitive Development Among Premature Infants

Previous Serial Number: None

Principal Investigator: Frank A. Pedersen, Ph. D.

Other Investigators: Michael S. Duchowny, M.D.

Cooperating Units: Sibley Hospital, Washington Hospital Center

Man Years:

Total:	1.1
Professional:	1.0
Other:	.1

Project Description:

Objectives: To study early parent-infant interaction and cognitive development among premature infants. The basic problem is to distinguish the effects of (a) the premature infant's isolation and separation from parents that is a consequence of being kept in the nursery, and (b) possible neurological damage. The study will involve comparisons between two groups of prematures that differ in degree of neurological risk (minimal and high) and a normal term control group. Within this framework, there will be an attempt to ascertain differential effects in the following areas:

1. The study and quality of the mother's interaction with her infant.
2. The parent's description of the infant's temperament and early personality.
3. The father-infant relationship.
4. The husband-wife relationship.
5. The infant's early cognitive and cognitive-motivational development.

Methods Employed: The sample will consist of 15 premature infants with minimal risk of neurological damage, 15 premature infants with a significant risk of neurological damage, and 15 normal term infants. Careful selection criteria have been worked out. The groups will be comparable on the following variables: birth order (all first born), socioeconomic level, ethnicity, maternal age, sex composition, and age from conception at time of data collection.

There are three phases of data collection that will be concentrated around the 5 month age period:

1. Mother-father interview. Both parents will complete a Q-sort type of inventory to characterize the infant's temperament and early personality characteristics. There will also be an inquiry into the father's relationship with the infant and some aspects of the husband-wife relationship.
2. Mother-infant observation. There will be one home observation lasting approximately two hours, utilizing categories and techniques developed by the Social and Behavioral Sciences Branch for studies of early mother-infant interaction. In addition, ratings will be made of some of the qualitative aspects of the relationship.
3. The infant's cognitive development will be assessed with three procedures: the Bayley Scale of Infant Development, a structured situational test of exploratory behavior and preference for novel stimuli, and a measure of habituation to visual stimuli.

Major Findings: The study is still in the planning stages. Additional work must be completed on various research techniques. Permission has been received to review nursery records to locate cases meeting all the criteria. At present a record review is in progress.

Significance to Biomedical Research and the Program of the Institute: In addition to the possibility of neurological damage, there are several potentially adverse environmental circumstances associated with prematurity. There has been speculation that the early separation from the mother in the nursery adversely affects her capacity to relate to the infant. Isolation of the premature in the nursery is also associated with significant restriction in sensory input, which might have an adverse effect. Little is known whether the father's relationship is different with prematures or whether the husband-wife relationship is affected such that the early environment is less facilitative of the infant's development. This investigation will contribute information in these areas on a problem of considerable social significance.

Proposed Course of Project: We expect to pilot test procedures by the end of FY '75 and begin formal data collection in FY'76.

Honors and Awards: None

NICHD ANNUAL REPORT
July 1, 1973 through June 30, 1974
Reproduction Research Branch

SUMMARY

The mission of the Reproduction Research Branch is the development of scientific data and theory basic to reproductive biology. To do this we have used techniques from such disciplines as physical chemistry, mathematics, biochemistry, reproductive biology, endocrinology and internal medicine. At the level of organization below the whole animal, existing areas of research include mathematical modeling of hormone action, the acquisition of new data about hormone-receptor interaction and protein-protein interaction, the mechanism of activation of the target cell and of the secretory process, and the regulatory phenomena governing secretory rates. In parallel with these basic studies are the investigations into hormonal aspects of the reproductive process in man and the experimental animal. A continuing interest and involvement in clinical problems are not only desirable but necessary since the results of study in man are the final arbiter of the relevance of results in the laboratory.

Because much of the current thrust in molecular reproductive biology is concerned with the protein hormones, this field has been particularly exciting. A newly described solid phase synthesis of peptides via α,β-unsaturated amino acids offers advantages over current methods. This work derives from studies of the α,β-unsaturated amino acids present in several membrane active antibiotics. The elucidation of these structures is a major achievement in peptide chemistry. Using the high resolution of NMR, it was shown that tyrosine and phenylalanine residues of lysine vasopressin are directly involved in neurophysin binding. The techniques of analytic polyacrylamide gel electrophoresis have been modified so that the gel systems can be used preparatively and be semiautomated. A theory of transient state of isoelectric focusing was expanded to give a new means for physicochemical characterization of proteins and polypeptides.

The solubilized gonadotropin receptor of the testis was shown to behave as an asymmetric molecule with a Stokes radius of 64 to 70A. The receptors contain minor lipid components necessary for gonadotropin binding. Disulphide bonds form an important component of the receptor. The dispersed Leydig cell system was improved so that it can serve as a sensitive bioassay system capable of giving independent estimates of gonadotropin potency. The affinity of receptors of rat and bovine adrenal cortex for angiotensin II was measured and the affinities were shown to be altered by guanyl nucleotides and sodium ions. The structural features of angiotensin II antagonists that confer specificity at smooth muscle receptors are those associated with antagonist activity at adrenal cortical receptors. These systems and others have been used to "fit" dose-response curves by a new method that makes it possible to evaluate the Hill coefficient, the most important parameter for describing and quantifying regulatory systems. In another hormone secretory system, the adrenal medulla, secretory vesicle membranes were shown to fuse with plasma membranes presumably as part of the secretory process. The activation of adenyl cyclase in these isolated membranes by agents such as carbachol suggests that activation is related to the secretory process.

The gonadotropins remain a major focus of interest. The ß-subunit has been isolated free of contaminants by polyacrylamide gel electrophoresis. The carboxy-terminal amino acid sequence that is unique to hCG ß-subunits has been used to immunize rabbits. The antiserum has complete specificity for hCG. In other studies, thyroid-stimulating activity has been shown to be intrinsic to hCG. A simple specific assay for mCG was developed. This will facilitate studies in primates since pregnancy can be diagnosed 18 days after fertilization. Reagents will be made available to interested scientists.

In continuing studies on follicular growth, estrogen was found to be necessary for maximal weight response to FSH and LH. Treatment with an antiestrogen during administration of gonadotropin increased the rate of follicular atresia. Intraovarian androgen is also important in the ovarian response to hCG. Administration of hCG to pseudopregnant rats caused a fall in cAMP-dependent protein kinase within 5 minutes of injection. Studies in rat testis yielded similar results.

In attempts to define the cell of origin of factor(s) regulating FSH secretion, two approaches were used. The germinal epithelium of rats was depleted by either Vitamin A deficiency or administration of hydroxyurea. In each case FSH levels did not increase due presumably to remaining normal Sertoli cell function. The persistent level of γ-glutamyl transpeptidase was further evidence that the Sertoli cell was unchanged. Initial studies of the androgen-binding protein of monkey and human testis showed that it was possible to distinguish it from TeBG by physical criteria and that it occurs as several charge isomers.

Clinical investigations have continued in several areas. Two types of patients with secondary amenorrhea were distinguished on the basis of response of gonadotropins to clomiphene citrate and LHRH. This will permit more rational therapy for infertility. The continuing exploration of hypothalamic dysfunction noted in women with anorexia nervosa has led to the finding some amenorrheic women also have aberrations of core temperature control, and mild abnormaliites of ADH release. This is the first evidence of hypothalamic disturbance in amenorrhea.

The fractionation of plasma and urinary hCG using specific antisera revealed that patients with molar pregnancy have only hCG in the fluids. By contrast, women with normal pregnancy or those patients with gestational trophoblastic disease not responding to chemotherapy have free and altered subunits present. An extensive comparison of plasma hCG with urinary gonadotropin has been carried out and the excellent correlation means that routine bioassays will no longer be necessary.

An extensive study of men with idiopathic oligospermia was initiated. High doses of FSH (Pergonal) have not modified sperm output or quality. From these and other data it is unlikely that FSH will be useful in therapy of men with oligospermia in contrast to current claims. In collaborative studies of infertile men, the Sertoli cell appears to be abnormal by electron microscopy. This is the first anatomic evidence of a Sertoli cell disorder.

In extensive collaborative studies, it was shown that patients with low-renin hypertension have the same susceptibility to strokes and heart attacks as those with normal or high plasma renin activity. The renin-angiotensin system was evaluated throughout pregnancy. Renin secretion and renin substrate levels increased during the first trimester. This is different from the effects of oral contraceptives which reduce renin secretion.

This year again we have been aided by Visiting Fellows and Guest Workers from Europe, Asia and South America. These scientists and our Clinical Associates have been a continued source of stimulation. The collaborative efforts within the Branch and with other scientists in Bethesda and elsewhere have strengthened the research and multiplied our effectiveness. Finally, the support of the Institute for the use of service contracts has enabled us to shift some of the burden of routine work and has thereby freed our staff for more creative research.

Serial No. HD-RPL-1
1. Reproduction Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biology of Reproduction: Clinical and Experimental

Principal Investigator: Dr. Mortimer B. Lipsett

Other Investigators: Dr. G.T. Ross, Dr. R. J. Sherins, Dr. D. Rodbard, Dr. A. Chrambach, Dr. H. Pollard, Dr. K. Catt, Dr. J. Vaitukaitis, Dr. M. D. Catt, Dr. G. Hodgen, Dr. B. Gulyas, Dr. W. Nixon, Dr. D. Loriaux, Dr. E. Gross, Dr. H. Chen, Dr. J. Cohen, Dr. M. Hayes, Dr. C. Cargille, Dr. P. Krueger, Dr. B. Nisula, Dr. W. Gerber, Dr. P. Fredlund, Dr. J. Hansen, Dr. R. Vigersky, Dr. S. M. Harman, Dr. J. Douglas, Dr. C. Mendelson, Dr. J. Janick, Dr. P. Hoffman, Dr. R. Easley, Dr. S. Saltman, Dr. O. Zinder, Mr. D. Ryan, Dr. G. Coffman, Mrs. M. Wyckoff, Mr. C. Turner, Mr. D. Barber, Mr. R. Reid, Mr. J. Brice, Mr. A. Baukal, Miss J. Brown, Mr. J. Morell.

Cooperating Units: Department of Anatomy, Harvard Medical School; Department of Obstetrics, Medical College of Virginia; Department of Physiology, Howard University; Massachusetts Institute of Technology; Dermatology Branch, NCI, Clinical Immunology Branch, NICHD; Arthritis and Rheumatism Branch, NIAMD; Laboratory of Microbiology, NIAID; Department of Chemistry, Harvard University; Washington University School of Medicine, St. Louis; Laboratory of Pathology, NCI; Laboratory of Physical Biology, NIAMD; Department of Biophysics, Johns Hopkins University; Laboratory of Mathematical Biology, NIAMDD; Department of Medicine, College of Physicians and Surgeons; University of California Medical Center; Department of Physiology, Case Western Reserve School of Medicine; DCRT; Department of Biochemistry, UCLA; Georgetown University School of Medicine; Pregnancy Research Branch, NICHD; Cleveland Clinic; Stanford University; Tubingen University; Laboratory of Chemistry, NHLI; CERN, France.

Man Years:

Total: 63
Professional: 41
Other: 22

Project Description:

Objectives:

1. To understand mechanism of hormone secretion and action;
2. To define hypothalamic-pituitary-gonadal interrelations in health and disease;
3. To explore the state of hormones, prohormones, and hormonal subunits in blood, and to assess such physiologic parameters as binding to plasma proteins, clearance, and volumes of distribution;
4. To study the regulation and biology of germ cell growth and maturation;
5. To characterize the interactions between the ovarian follicle or seminiferous tubule with FSH.

Methods Employed:

A great variety of techniques are employed since the research encompasses such widely divergent areas as mathematical biology, protein hormone chemistry and physiology, steroid biochemistry, morphology, and clinical investigation. New methods that have been either developed or adapted during the year are:

Non-enzymatic cleavage techniques for structural elucidation of peptide structure;

Automation of preparative polyacrylamide gel electrophoresis;

General method of protein fractionation by SDS-polyacrylamide gel electrophoresis;

Angiotensin II binding to membranes of adrenal cortical cells;

Development of an assay for mCG in urine;

Development of an antiserum completely specific for hCG and of improved sensitivity;

Utilization of Vitamin A deficiency to study effects of depletion of germinal epithelium;

Measurement of rat urinary FSH and LH;

A fast method for measurement of urinary estriol glucuronide for use in monitoring high-risk pregnancy;

A new method for fitting dose-response curves that permits simultaneous evaluation of Hill coefficients and maximal response.

Major Findings:

I. Protein Chemistry

A catalogue of physical-chemical states of hGH is being constructed, comprising "big" and "big big" hGH, iodo-hGH, desamio-HGH's, aggregation states, hGH from tumor-tissue culture, etc. The plasma catalyzed bioactivation of hGH was shown to result in increased hGH potency. Crude (2-3,000 IU/mg) commercial hCG has been purified preparatively in one step by selective restacking at pH 5, giving rise in good yield to a preparation of 15,000-18,000 IU/mg which is as good as any other presently available purified preparation. The ß subunit has been prepared in milligram amounts free of hCG (less than 3 IU/mg), using selective stacking.

Human prolactin (hPRL) prepared from amniotic fluid, after dealbumination and isoelectric focusing, yields a mixture of four isohormones of hPRL, in about 50% yield. The preparation, however, remains contaminated with Ampholine. Accumulation of sufficient amounts of hPRL is in progress that would allow deampholination and separation of the four isohormones from each other for the purposes of producing isohormone-specific radioimmunoassays. We have succeeded in iodinating hPRL (from amniotic fluid) without changing its conformation. The iodinated product is therefore suitable for physiological studies and use as a marker in fractionation. Evidence for conformational integrity based on K_R-Y_o -ellipses is unique and should serve as a model for iodoprotein preparations in general.

Androgen binding protein of human testis was identified physico-chemically. It appears in PAGE at pH 8 as a single molecular unit existing in various aggregation and charge isomeric forms. One of these forms is indistinguishable from TeBG. Discrimination between hABP from hTeBG is being attempted on the basis of binding characteristics, determined from PAGE data by computerized peak location and peak integration methods.

Preparative PAGE has been automated and band width determinations of a specific protein, buffer system and gel allow for prediction of resolution in preparative PAGE. An instrument was constructed capable of taking several hundred band width measurements during a single gel electrophoresis experiment. Computer programs were written to give objective peak recognition and band width at half-peak height as a function of migration distance. Systematic tests of the parameters affecting band width have been started with standard proteins. A general method of protein fractionation by SDS-PAGE with stacking has been developed. Present SDS-PAGE methods either do not provide automatic sample concentration by stacking, or contain hidden secondary moving boundaries formed by the trailing ion and the detergent. The physical conditions behind these secondary boundaries are unknown. A multiphasic zone electrophoresis system was found that is not in practice perturbed in this fashion, and that does allow for stacking of SDS-proteins. Ferguson plots of standard proteins are being constructed for this system to provide a general method of molecular weight computation for protein subunits. The theory of the transient state of isoelectric focusing was expanded and tested in a preliminary fashion. This theory makes it possible to estimate the diffusion coefficient and an "isoelectric focusing parameter", pE for protein and polypeptide hormones. It thus provides a new means of physico-chemical characterization of protein and polypeptide hormones and a basis for computer optimization of conditions for preparative isoelectric focusing. A computerized system for automatic "peak detection" and calculation of moments was developed (for analysis of overlapping Gaussian curves). Improved statistical methods and computer programs were developed for calculation of molecular weights using gel filtration or SDS-polyacrylamide gel electrophoresis methods. These have been applied to a wide variety of protein and polypeptide hormones and also to Rhodopsin. Virtually all labs using SDS-PAGE and gel filtration use no statistical analysis whatsoever. The methods developed here should gain widespread acceptance, provide realistic estimates of 95% confidence limits for MW determinations, and modify the common experimental approach.

II. Mechanism of Hormone Action:

Characterization of soluble gonadotropin receptors and hormone-receptor complexes by gel filtration and sucrose density gradient centrifugaation revealed the presence of several physical forms. In addition to the 6.5S form of the free receptor extracted by Triton X-100 and the 7.5S hormone-receptor complex formed by hCG binding to the free receptor, more rapidly sedimenting forms were observed after dialysis of the hormone-receptor complex and Triton extraction of preformed hormone-receptor complexes. In both cases, the sedimentation constant of the resulting hormone-receptor complexes was 8.8S, instead of the 7.5S form derived by equilibration of the free solubilized receptors with hCG. Extraction of preformed hormone-receptor complexes from testis particles previously equilibrated with ^{125}I-hCG was also performed with Lubrol PX, Lubrol WX, and sodium deoxycholate. With these detergents, hormone-receptor complexes sedimenting at 7-7.5S were obtained. No significant variations in the density of the complexes extracted under these various conditions were observed. The multiple forms of the hormone-receptor complex extractable by various detergents is attributed to a combination of factors, including the highly asymmetric nature of the receptor molecules, the presence of a minor lipid component and carbohydrate components and probably a degree of differential binding of the individual detergents. In addition to these observations of the gonadotropin receptors of the rat testis, comparable findings have been obtained during solubilization of gonadotropin receptors of the rat ovary. Marked similarities exist between the detergent extracted species demonstrated in the rat ovary, and those derived from the interstitial cells of the rat testis. Such findings indicate that the structural properties of the two gonadal receptors are probably closely similar.

Gonadotropin receptors extracted with detergents from both testis and ovary show a detectable loss of binding affinity by comparison with the original particulate form. In general, the binding affinity of the solubilized receptors is about 50% of that observed in the particulate receptors. During storage, the solubilized free receptors undergo progressive degradation with time, resulting in a major loss of binding activity within two or three days at 4° C. Serial determination of the binding characteristics of solubilized receptors during storage at low temperature has revealed that this marked loss of binding capacity is accompanied by the appearance of a small quantity of high affinity binding sites, previously undetectable against the lower average affinity constant of the majority of the solubilized receptors. Thus, preferential degradation of the predominant lower affinity sites reveals the presence of a small proportion of sites with high affinity equal to that of the original particulate receptors.

Exposure to trypsin results in a major loss of activity in both particulate and soluble receptors, indicating that protein forms a major component of the receptor site. In intact cells (see below) the effects of trypsin are biphasic with initial enhancement of binding activity. Neuraminidase does not diminish binding of gonadotropin to receptors and, in fact, causes a slight enhancement of hormone uptake. Treatment with phospholipase A and phospholipase C has complex effects upon binding. In general, phospholipase A markedly impairs the ability of free soluble or particulate receptors to bind gonadotropin during subsequent incubation, though the enzyme has relatively little effect upon preformed hormone receptor complexes. Phospholipase C has little or no effect upon the free receptors and causes aggregation of the 8.8S hormone receptor complex. The lack of effect of phospholipase A upon preformed hormone receptor-complexes suggests that binding activity is significantly influenced by an important phospholipid component of the binding site.

The specific binding of ^{125}I-hCG by receptors of the rat testis interstitital cells was not reduced by alkylation of free -SH groups in either particulate or solubilized receptor preparations. However, reduction and alkylation of receptor disulphide groups almost completely abolished subsequent gonadotropin binding by the uncharged receptors sites, but had little effect upon the retention of bound hormone by preformed hormone receptor complexes. Reduction and alkylation of the gonadotrophic hormone was also shown to impair binding of hCG to testis receptors. These results indicate that free -SH groups are not necessary for specific binding of gonadotropin to testis receptors, whereas disulphide bonds formed an important component of the receptor and appear to be essential for hormone receptor interaction.

Treatment of dispersed Leydig cell with low concentration of trypsin has been shown to enhance gonadotropin binding. This effect of trypsin is biphasic and is followed by subsequent decline in binding to very low levels after prolonged exposure to trypsin. These findings provide a useful approach to the analysis of the relationship between the quantity of binding sites per cell and the sensitivity and subsequent responsiveness of the cell to given concentrations of gonadotropin. In addition, this phenomenon has applications to studies on the turnover of gonadotropin receptors if re-emergence of receptors from enzyme-treated cells can be analyzed under appropriate conditions in vitro.

Further studies on the responses of dispersed Leydig cells to gonadotropin stimulation in vitro have shown that the sensitivity to gonadotropin can be considerably enhanced above that observed with the intact rat testis system. Thus, particularly in the presence of phosphodiesterase inhibitors, dose response curves can be obtained with hCG over the range between 0.1 and 1 mU per incubation vial. Comparable results are obtained with human and ovine LH. Thus, the dispersed cell system has the capacity to respond to quantities of gonadotropin which are normally present in plasma and is currently being applied to the bioassay of LH activity in the plasma of normal and postmenopausal subjects. This method provides for the first time a simple and sensitive assay system with the facility for measurement of gonadotropin concentrations in plasma by bioassay.

Since the protein and polypeptide hormones exert major effects at the plasma membrane, the chromaffin granule membrane has been used as a model for study of activation and secretion. It has been found to possess at least four glycoproteins; one of these is dopamine-ß-hydroxylase (72,000 MW). The major glycoprotein-staining species is a 102,000 MW protein which can be extracted from purified membranes with lithium iodosalinylate. Plasma membranes have been isolated from adrenal medulla tissue. These membranes also have markers for secretory vesicle membranes such as DBH, and polypeptide chains defined by SDS gels. A potent adenyl cyclase in this preparation has been studied. It is activated by carbachol, an agent capable of stimulating release of catecholamines from intact granules. Calcium is able to inhibit adenyl cyclase activity by 97%, with an apparent $K_i = 30\mu M$. This reciprocal effect may be relevant to the regulation of secretion. The adrenal medulla was found to have a large quantity of myosin, a muscle protein (1 gram/50 grams tissue). The protein has been purified by column chromatography and characterized by SDS gel electrophoresis. The light chains appear to have a lower molecular weight than those from skeletal muscle. Isolated chromaffin granules are capable of active transport of epinephrine. The uptake process appears to be composed of either two kinetic components, or is negative cooperative with respect to substrate. This transport system is the specific target of reserpine and related drugs. The mechanism of action of chloropromazine (CPZ) has been studied using squid brain synaptosomes. CPZ was found to competitively inhibit the uptake of L-noradrenaline (L-NA) and 5-hydroxy tryptamine (5 HT) but not four other putative transmitters. The K_i of CPZ for L-NA and 5 HT was found to be 20-fold lower than the affinities of the substrates for the uptake systems. It is concluded that a specific CPZ-sensitive site is common to the uptake mechanisms of L-NA and 5 HT.

A new method for "fitting" dose response curves has been developed (e. g. for cAMP or corticosterone production by the adrenal in response to ACTH or for cAMP or testosterone production by the testis-or Leydig cell- in response to hCG or LH). This method uses weighted iterative non-linear regression to find optimal values for a 4-parameter logistic model. This makes it possible to evaluate Hill coefficients and maximal response simultaneously for a variety of biological systems. Results indicate apparent "positive" cooperativity in the action of hCG, ACTH, and several analogues of ACTH; Hill coefficients for cAMP, corticosterone production, and/or testosterone production are significantly greater than unity. The methods developed here should provide a useful tool for analysis of data from a wide variety of endocrine systems. Computer simulation studies were used to evaluate the relationship between the Hill coefficient and the "degree of cooperativity" in "stepwise" models. Also, factors affecting the linearity of the Hill plot were evaluated. These model studies should provide a useful guide to experimentation. In particular, one can study the effect of ions (Mg^{++} Ca^{++}, nucleotides (ATP, cGMP etc.) on the shape of the dose response curve, with respect to the Hill coefficient, which is one of the most essential parameters to describe and quantify regulatory systems. Factors which affect n_H are likely to be interacting directly with the allosteric enzyme as other regulatory macromolecules. Until the n_H is determined, it is impossible to build even the most simplistic model. Potentially, n_H can give information about the number of subunits. Ultimately, when receptors are isolated, it is intended to apply more advanced methods developed by the enzymologists to study kinetics of hormone-receptor systems.

Efforts have been made to delineate the role of protein kinases in hormone action. Ovaries of pseudopregnant rats were homogenized and the protein kinase fraction partially purified. Cyclic AMP dependent protein kinase activity decreased significantly within 5 minutes of injection of hCG and usually fell to a nadir at 30 minutes. The cAMP-dependent P-K activity was maximally suppressed with less than 0.5 IU hCG per animal in contrast to endogenous increasing cAMP levels up to 5 IU hCG per animal. This disparity of the sensitivity of response between P-K and cAMP activation suggests that protein kinases may protect the cell from hyperstimulation.

Studies in the rat testis have yielded the same qualitative observations for hCG action. A limited number of studies relating protein kinase activation with FSH stimulation have been carried out. The number of binding sites for FSH appear to be far fewer in the ovary and testis and have limited more extensive studies up to this time. Optimization of the P-K purification system has been attained for the hCG studies and should now allow us to carry out more extensive studies with FSH in the near future.

Molecules of hCG β or hTSH β were hybridized with bTSh$_{\alpha}$, since the α subunits do not cross react between species and assuming the conformation of the β subunit is different whether it is in isolated form or combined with a complementary α subunit.

Determination of the structure of hCG has facilitated several new studies. The carboxy-terminal amino acid sequence that is unique to the hCG β subunits was conjugated to BSA and used to immunize rabbits. Antisera produced have been used in both radioimmunoassays and hemagglutination inhibition assays for hCG in serum and urine from pregnant women, women with trophoblastic neoplasms, and men and women with other nontrophoblastic tumors that secrete CG.

Maintenance of a colony of vitamin A deficient male Holtzman rats has continued to provide an excellent animal model of reversible germ cell depletion. Even after 18 months of Retinol deficiency (with Retinoic acid diet supplementation), the germinal aplasia is reversible and within 70 days of Retinol supplementation, fertility is completely restored. Thus, long term maintenance of such a colony has and will continue to provide sufficient animals for long term studies of seminiferous tubular physiology.

Even after long term Retinol depletion, the pituitary axis is responsive to castration with appropriate marked increases in both plasma FSH and LH concentrations. Nevertheless, in spite of 90% loss of testicular mass and complete germinal aplasia, no increase in FSH concentrations is evident. These long term studies provide additional support for the concept of independent regulation of FSH by factor(s) from the Sertoli cell per se. In another approach to this problem, the drug Hydroxyurea was used to deplete the germinal epithelium. This chemotherapeutic agent is toxic only to cells undergoing DNA synthesis. The FSH levels were unchanged during 70 days of treatment, due presumably to persisting normal Sertoli cell function.

Measurement of rat urinary FSH and LH has been achieved using gel filtration to desalt aliquots of urine and subsequent direct radioimmunoassay of the filtrate to assess titers of FSH and LH. The method appears to be accurate and precise. Urinary excretion of these gonadotropins parallels concentrations of FSH and LH in plasma and permits specific assessments without the technical requirements for venipuncture in small animals. This will permit the use of the rat for bioassay of the putative FSH-regulating factor derived from the tubule.

The thyroid stimulating (TSA) and follicle stimulating (FSA) activities of the crude precursor hCG preparations and the highly purified hCG extracted from these are being studied. The results are consistent with the hypothesis that the TSA is intrinsic to the same molecule as that having the luteinizing hormone activity (LHA) in hCG preparations. In contrast, studies of the FSA of hCG in estrogen primed hypophysectomized immature female rats is consistent with the hypothesis that the FSA and LHA are separable. Isolation of the substance with FSA will be pursued.

It has been shown, for the first time, that the anti-estrogen, clomiphene inhibits ovarian weight responses of hypophysectomized immature female rats to mixtures of FSH and hCG. More importantly, treatment with the anti-estrogen increases rates of follicular atresia in these animals.

During the course of studying the hCG assay in estrogen primed hypophysectomized, immature female rats, we observed that doses as low as 0.1 IU (10 nanograms) of highly purified hCG inhibited the ovarian weight response of these assay animals to exogenous estrogens. It has now been shown that simultaneous administration of anti-testosterone serum inhibits this effect of low doses of hCG. These observations may lead to increased understanding of the roles of intraovarian estrogens and androgens in controlling follicular maturation and atresia and in turn to new methods for fertility control.

Clinical Investigation:

Patterns of change in FSH and LH concentrations in first-morning urine specimens were compared with those in plasma samples collected at 8:00 a.m. and in consecutive 24-hour urine specimens collected between two successive menstrual periods from eight normal young women. Results showed a highly significant correlation, indicating that first-morning urine specimens, easier to collect than blood or 24-hour urine specimens provide satisfactory information concerning changes in daily pituitary gonadotropin secretion.

Sixty consecutive first-morning voidings were then collected from pre, per- and post pubertal girls, and immunoreactive FSH and LH concentrations have been measured in these specimens. In two of five pre-pubertal (P) girls studied, patterns of FSH excretion were found to be similar to those seen in postmenarchal girls with 28-30 day periodicity.

We will continue to measure the more than 2500 such specimens collected and processed already. In addition, a second series of specimens will be collected from these same girls one year later during which time pubescence will have advanced in some of them. These data should provide the first long-term continuous and periodic longitudinal studies of these aspects of puberty in girls.

The Male Infertility Clinic continues to grow. Approximately 50 new patients per year are seen in the Outpatient Department alone. These patients provide a wealth of material for study of testicular physiology. Importantly for the patient, it provides one of the few competent diagnostic facilities for evaluation and management of disorders of male reproductive function.

The ever expanding data from semen analysis and steroid and gonadotropin assays are now stored in a WYLBUR Data Retrieval System. Detailed evaluation is now underway of the various parameters of semen analysis in order to define the minimal characteristics of normal semen. Such an analysis is critical because of increasing evidence that reduction in sperm count per se does not correlate with reduced fecundity.

Analysis of pituitary and Leydig cell function among infertile men with severe germ cell loss has demonstrated both a small but significant increase in LH levels and an increase in responsiveness to LHRH. The data suggest that a compensated Leydig failure co-exists when FSH levels are markedly increased due to severe loss of germ cells. This Leydig cell failure is not evident clinically. Furthermore, it is not clear whether the Leydig cell is directly involved by the process producing the seminiferous tubular injury or whether the tubular structures normally contribute to Leydig cell steroid secretion.

Thus far, administration of high dose Pergonal (FSH) to 20 men with idiopathic infertility has not succeeded in increasing or modifying sperm output or semen quality. In view of our earlier data that sperm output can be maintained despite marked reduction in FSH levels, it would appear that FSH therapy has no role in treatment of oligospermia. Further studies are in order before such a statement can be made unequivocally.

Preliminary evaluation of testicular biopsy material from infertile men shows ubiquitous vacuolization of Sertoli cells. This is the first anatomical evidence of Sertoli cell disorder. Further detailed ultrastructural analysis is underway.

Women presenting with primary or secondary amenorrhea as well as oligomenorrhea have continued to be evaluated for abnormalities of pituitary-hypothalamic dysfunction. We have found:

a. Amenorrheic women failing to respond to clomid, reflected by no significant increase in peripheral gonadotropin concentration, have unusual response to exogenous LRF. This group of patients secretes essentially equal quantities of FSH and LH in response to LRF. Moreover, the integrated FSH response is greater than that observed in normally cycling women tested in either the early or late follicular phases and greater than that observed in amenorrheic women who respond to clomid and are given the same dose of LRF.

b. A significant number of amenorrheic or oligomenorrheic women have aberrations of core temperature control and mild abnormalities of ADH secretion.

c. When women with oligomenorrhea are tested with LRF within the first three days of the follicular phase, an excessive release of LH and FSH is observed, relative to spontaneously cycling normal women tested at that time. In essence, this group of women is exquisitely sensitive to LRF.

Women who have had full endocrinologic evaluation for infertility and desiring pregnancy are treated in our fertility clinic. Ovulation induction is carried out in those women without organic pelvic disease but have documented endocrinologic aberrations of reproduction. An approach simulating the normal cyclic release of pituitary gonadotropins is used and, to date, this approach has resulted in 10 consecutive single pregnancies. A large number of observations is needed to prove that this approach is superior to those of previous studies in which 25% of patients undergoing ovulation induction with Pergonal have had multiple births.

Patients with gestational trophoblastic disease continued to be studied for:

a. The correlation of changes in immunologic (specific hCG RIA) and biologic (MUW activity) activities prior to, during, and after chemotherapy. Results to date show an excellent correlation between those activities, obviating the tedious bioassay procedures used for monitoring this group of patients.

b. Correlating the forms of hCG in plasma, urine and tissue extracts with their responsiveness to chemotherapy. Previous studies have been extended to extracting molar tissue and characterizing the forms of hCG present. In short, no free hCG subunits are present in contrast to the profiles found in placentas of normal pregnancy and tissue extracts of patients with gestational trophoblastic disease not responding to chemotherapy.

In continuing studies of steroid hormones, we found that estradiol does not bind to TeBG at 37° C. This observation clarifies many apparently contradictory data about testosterone:estradiol ratios and physiologic effects. A simple quick assay for urinary estriol glucuronide was developed that will be valuable in following high-risk pregnancy. In order to evaluate the transformation of testosterone to dihydrotestosterone at the target tissue in man, specific assays have been devised for the androstanediols, the metabolites of dihydrotestosterone.

Significance to Biomedical Research:

In the effort to prepare protein hormones of high quality, new methods of protein fractionation have been developed that have proved useful to biochemists around the world. The mathematical treatment of these techniques and of radioimmunoassays has systematized these procedures internationally. The studies of polypeptide hormone receptors indicate some of the initial steps of hormone action, a necessary prelude to modifying effects of hormones. In the gonadotropin area, the fundamental studies of subunit physiology have led to production of antisera capable of distinguishing molar and normal pregnancy. In addition, the first adequate monkey pregnancy test was developed which will be valuable to those using timed pregnancies in the monkey. In clinical investigation we have defined parameters of male fertility and evaluated the usefulness of a current therapeutic regimen.

Proposed Course for the Branch:

In the areas of research concerning hormonal regulation of the ovarian follicle and seminiferous tubule, basic biology is needed. This emphasizes the long-felt need for developing biology within the Branch. Cell kinetics, differentiation, and division now become an important segment of the study of the process of regulation.

We will continue to explore those areas of biochemistry and physiology that relate to basic reproductive processes. Of greatest importance will be the attempts to proceed beyond the binding step of peptide hormone action to the associated membrane events. New physical tools will be needed for these studies.

In the clinic, the study of male fertility is an integral part of efforts to understand spermatogenesis. Intensive study of the androgen-binding protein of the Sertoli cell may prove rewarding in defining and possibly altering one important process of spermatogenesis.

Serial No. HD-RPL-11
1. Reproduction Research Branch
2. Section on Hormonal Regulation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mechanism of Action of Protein and Polypeptide Hormones

Principal Investigator: K. J. Catt

Other Investigators: J. Douglas, A. Baukal, P. Fredlund, J. M. Ketelslegers

Cooperating Units: Georgetown University Medical School; Cardiovascular Research Lab of D. C. General Hospital; M. Bumpus Division of Research, Cleveland Clinic, R. Chez, Pregnancy Research Branch, NICHD

Man Years:

Total: 5
Professional: 5

Project Description:

Objectives:

1. To analyze the mechanism of action of polypeptide hormones;
2. To examine the effects of oral contraceptive steroids on the renin-angiotensin system and on other physiologic responses.

Methods employed:

The putative location of gonadotropin receptors in the cell membrane has been further verified by sucrose density gradient centrifugation of particulate testis binding fractions, demonstrating that binding activity is associated with a membrane-containing region which is rich in 5' nucleotidase. Analysis of kinetic and equilibrium binding data derived from particulate and soluble receptors has been facilitated by the use of a computer modeling system (MLAB) for direct analysis of binding data with fitting to appropriate expressions for kinetic and equilibrium binding conditions. Such expressions have been modified to include terms for degradation of receptor sites and hormone during incubation and have permitted graphical analysis of complex binding data to be performed with improved speed and accuracy. Corrections for the maximum binding activity of labeled hormone and the specific activity of the tracer in terms of bio- and receptor-binding activity, have also been employed. The binding constants derived by these methods have been in good agreement with those obtained earlier by less sophisticated methods and of considerably improved precision. Derivation of association rate constants by this method over a wide range of hormone and receptor concentrations has given a constant value of $3.1 \times 10^{7} M^{-1} min^{-1}$; the kinetics of the hormone-receptor interactions are those of a second-order chemical reaction.

Fractionation of soluble gonadotropin receptors by polyacrylamide gel electrophoresis has been performed in detergent-containing gels, and optimum running conditions and staining procedures have been developed for studies on the active soluble binding protein. This system will be applied to determination of the physical properties of the complex during electrophoresis, and to monitor the purity of receptors during further isolation from membrane binding fractions.

Initial studies on the purification of soluble receptors by affinity chromatography have been performed and have indicated that isolation of gonadotropin receptors will be possible by this technique. Optimal conditions determined during pilot studies on the rat testis will be applied to purification of receptors from large quantities of cell membranes obtained from the bovine corpus luteum, rat ovary and rat testis.

Mechanism of action of LH

The action of LH/hCG upon the Leydig cell has been further studied. Preparation of collagenase-dispersed Leydig cells has been extensively investigated and a marked influence of collagenase batches upon cell viability has been detected. The relations between gonadotropin binding, cyclic AMP production and testosterone synthesis have been examined in greater detail and the dissociation between cAMP formation and testosterone production at physiological gonadotropin levels has been unequivocally demonstrated in the isolated cell system. The in vitro response of dispersed Leydig cells to LH and hCG has also been shown to provide a specific bioassay of extremely high sensitivity for these gonadotropins and has been applied to the bioassay of plasma levels of LH and hCG. The system is presently sensitive to hCG over the range 0.1 to 2 mIU per assay vial and detects LH in the plasma of normal and postmenopausal subjects.

Further studies on the dispersed Leydig cells will include detailed examination of the rate of biosynthesis of cAMP from labeled precursors during stimulation with levels of hCG which elevate testosterone formation without affecting total cyclic AMP production. The relationship between hormone binding and protein kinase activation will be studied, and the effects of metabolic inhibitors upon cAMP formation, protein synthesis and androgen synthesis will be evaluated. The general aim of these studies is to clarify the mechanisms by which gonadotropins stimulate androgen synthesis in the Leydig cell. The dispersed cell system will also be applied more extensively to the bioassay of plasma and urinary LH and hCG under a variety of physiological and pathological conditions and will be correlated with the results of radioimmunoassay of these hormones.

Angiotensin II Receptors

Binding sites for angiotensin II in the bovine and rat adrenal cortex have been further characterized by kinetic and equilibrium binding studies and have been confirmed to be located in the cell membrane by continuous density gradient centrifugation and assay of marker enzymes. The affinity of adrenal receptors for angiotensin II has been found to be significantly influenced by guanyl nucleotides and by sodium ions. Guanyl nucleotides decrease the equilibrium constant of the receptors by increasing the dissociation rate constant of angiotensin II, and sodium ions cause the appearance of a second-order of high-affinity sites during equilibrium binding studies.

Comparable effects of sodium ions upon angiotensin receptors have also been observed during treatment of rats with high and low sodium diets, with subsequent measurement of adrenal cortex binding sites and affinity in vitro. High sodium diet appears to increase the binding affinity of adrenal receptors for angiotensin II, while low sodium diet results in an increased number of lower-affinity binding sites. Plasma renin activity and plasma aldosterone were considerably increased after low sodium diet and were suppressed after high sodium diet. High potassium intake caused a more marked and consistent rise in plasma aldosterone than after low sodium diet and was devoid of effect upon plasma renin activity.

The affinity of rat adrenal receptors for angiotensin II was shown to be consistently higher than that of the bovine adrenal receptors. Purification of rat adrenal plasma membranes by sucrose density gradient centrifugation has given binding preparations of considerably improved storage properties, with higher binding capacity and much less degradation than the original adrenal homogenate fraction. The sodium effect has also been demonstrated in such membranes, which promises to be of great value for further studies on the rat adrenal receptors.

The adrenal cortex receptors have also been applied to extensive binding-inhibition studies with angiotensin II analogues, in particular, those which act as competitive antagonists of angiotensin II. A wide variety of antagonists, prepared by Dr. M. Bumpus (Cleveland Clinic, Cleveland, Ohio) have been evaluated and adrenal binding compared with antagonist activity in other biological response systems, partricularly smooth muscle. These studies have shown that structural features which confer antagonist activity at smooth muscle receptors are also associated with high binding-inhibition potency in the adrenal cortex; as in muscle, the most effective antagonists are those in which the C-terminal phenylalanine residue of angiotensin II is replaced by alanine or isoleucine. These studies have also demonstrated that the enhanced antagonist activity of sar^1 ile^8 angiotensin II is due to increased binding of the sar^1 derivative. Sar^1 angiotensin II also shows enhanced binding activity by comparison with angiotensin II and has recently been reported to exert increased agonist activity on blood pressure in vivo.

For studies on the mechanism of action of angiotensin II on aldosterone secretion, cell suspensions have been prepared from the bovine and dog adrenal cortex. This has been a difficult technique to standardize, but at present a collagenase-digested cell dispersion from dog adrenal is giving satisfactory steroidogenic responses to angiotension and ACTH. Steroid production is determined by direct radioimmunoassay of cortisol and aldosterone produced during incubation with LH 20 column fractionation where necessary.

Further studies on angiotensin II receptors will include localization to specific cells of the adrenal cortex, solubilization of the membrane-associated receptor, and investigation of the relation between peptide hormone binding, adenylate cyclase activation and aldosterone synthesis. The actions of angiotensin II must be correlated with those of ionic changes and ACTH.

Renin-angiotensin and Aldosterone Studies in vivo

Methods for assay of plasma renin parameters and angiotensin II have been modified and improved, and an assay technique for plasma aldosterone was established and validated. Plasma renin activity (PRA) measured in a large number of hypertensive patients (D. C. General Hospital) has confirmed the initial observation that patients with low-renin hypertension have the same susceptibility to strokes and heart attacks as those with normal or high PRA. Renin levels are not a contributing factor to such hypertensive complications; high renin values are more common in patients with severe hypertension but represent the result of renal impairment rather than the cause of the atheromatous vascular complications of essential hypertension. The response of low-renin patients to various forms of therapy has also been evaluated, and the elevated blood pressure in such patients has been shown to respond satisfactorily to diuretics or spironolactone.

The renin-angiotensin system and aldosterone secretion have been studied in a group of normal pregnant women. Assays for plasma renin substrate, renin concentration, renin activity, angiotensin II, and aldosterone were performed in about 50 subjects. Renin substrate levels and renin secretion became elevated while angiotensin II and aldosterone remained high throughout pregnancy. In a group of women taking oral contraceptives, renin substrate was similarly elevated; but renin secretion was reduced, PRA and angiotensin II was slightly to moderately increased, and plasma aldosterone was normal.

In collaboration with Dr. R. Chez, maternal and fetal PRA have been studied during pregnancy in the sheep, and responses to standard diuretic stimuli were evaluated. Fetal renin levels are consistently higher than maternal levels and respond irregularly to diuretics. Maternal PRA values do not rise until near the end of pregnancy and show consistent responses to diuretic administration. These studies will be extended to investigate the control of maternal and fetal renin secretion and aldosterone regulation during pregnancy in the sheep.

Serial No. HD-RPL-5
1. Reproduction Research Branch
2. Section on Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Analytical Techniques and Instrumentation
- a. Amino Acid Analysis
- b. Automatic Monitoring of Chromatographic Experiments

Previous Serial Number: HD-LB3

Principal Investigator: E. Gross

Other Investigators: J. Brown
J. L. Morell

Cooperating Units: Systems Maintenance Section DRS-BEI, DCRT
Pharmaceutical Development Service, Pharmacy Department, CC
Laboratory of Microbiology, NIAID

Man Years:

Total: 2
Professional: 2

Project Description:

Objectives

1. Determination of the amino acid composition of peptides and proteins;
2. Improvement of existing amino acid analyzers and automation of techniques otherwise requiring many time-consuming operations;
3. Adaptation of conventional amino acid analyzers to the separation and quantitation of unusual amino acids of microbial origin;
4. Design, development, and operation of automated multiple sample applicators, the final instrument to offer adequate capacity for the continuous operation for prolonged periods of time without attendance.

Methods Employed:

Ionexchange chromatography on custom resins of spherical shape, colorimetry, and electronic data processing equipment.

Major Findings:

A second modified and improved automatic sample applicator now operates on a 24-hour basis and performs 4-6 amino acid analyses depending upon the mode of operation. A third applicator is presently under construction. These applicators greatly facilitate the analysis of large numbers of peptide hydrolysates as they are derived from numerous fragments of various peptides under structural investigation. The amino acid analysis of nisin, subtilin, cinnamycin, and duramycin requires special conditions of column operation and adaptation to the presence of rarely encountered amino acids, such as hydroxyaspartic acid and lysinoalanine in cinnamycin and duramycin.

The amino acid analysis of samples of limited quantity is now possible on an instrument with electronic signal amplification. Amino acids are detected and determined at levels of less than one nanomole.

The stability of solutions of essential amino acids is impaired by the addition of bisulfite, until now advocated as a preservative free from side effects. Tryptophan is converted to at least five different products separable on the amino acid analyzer. Bisulfite containing tryptophan solutions cause changes in enzyme levels in rats and produce morphological alterations.

The cell wall proteins of Candida albicans contain unusual amino acids that are possibly derived from the ß-elimination products of ß-substituted amino acids by the addition of omega-amino acids.

Total number of amino acid analyses performed on instruments of the Section on Molecular Structure: 18 36

Significance to Biomedical Research and the Program of the Institute:

The structural elucidation of peptides and proteins requires large numbers of amino acid analyses. The extensive use of the amino acid analyzers with automatic sample application considerably facilitated the structural elucidation of nisin, subtilin, cinnamycin, and duramycin, all peptides with unusual amino acids. Unique degradation products of these peptides are also quickly determined by related approaches. Nisin and fragments of nisin are capable of inducing fetal resorptions in rats. The precise knowledge of the mode of action of these peptides will only be possible after characterization of the active peptides with the help of numerous amino acid analyses.

Purity criteria of synthetic peptides, e.g. hormone releasing factors, are established with the help of numerous amino acid analyses.

The homogeneity of essential amino acid solutions is of vital importance to the patient dependent upon hyperalimentation.

Proposed Course of Project:

(1) Refinement of the design features of the applicators. Amino acid analyzers equipped with automated sample applicators will be linked to small independent computers capable of feedback and process control in order to

demonstrate advanced techniques of laboratory automation and their application to protein chemistry. (2) The hydrolysis of peptides and proteins will be automated and samples directly advanced to the automatic sample applicator for column loading. (3) Other laboratory processes will be further automated in collaboration with the Division of Computer and Research Technology. (4) The sequence analysis of peptides and proteins will be automated (a) by utilizing solid supports and (b) employing mass spectroscopic techniques. (5) Sensitivity in amino acid analysis will be further increased by (a) colorimeter modification inclusive of constant temperature accomodation; (b) the use of ionexchange columns of lower diameter; and (c) modifications in reagents and buffers and their adequate accommodation.

Serial No. HD RPL-6
1. Reproduction Research Branch
2. Section on Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Synthesis of Biologically Active Peptides

Previous Serial Number: HD-LB5

Principal Investigator: E. Gross

Other Investigators: K. Noda
S. Matsuura

Man Years:

Total: 2-1/2
Professional: 2-1/2
Others: 0

Project Description:

Objectives:

1. Synthesis of peptides with biological activity, such as:
   a. effects on fetal development, and
   b. hormone releasing properties;
2. Testing and evaluating the biological properties of synthetic peptides.
3. Development of novel approaches to the synthesis of biologically interesting properties.

Methods Employed:

(1) Conventional techniques of peptide synthesis, (2) solid phase methods of peptide synthesis, (3) chromatographic and countercurrent distribution techniques for separation and purification, (4) animal experiments and bioassays in the biological evaluation of the synthetic products, (5) radioimmunoassays in screening procedures; (6) nuclear magnetic resonance and mass spectroscopic analysis of peptides; (7) novel routes to the synthesis of peptides, particularly peptide amides that possess hormone releasing properties.

Major Findings:

Synthesis via solid phase techniques of: Luteinizing hormone-follicle stimulating hormone releasing factor (LH/FSH-RF); H N-terminal tripeptide amide of LH/FSH-RF; the peptide has less than 0.1% of the activity of the natural releasing factor.

The Dehydroalanine Resin Technique of Solid Phase Peptide Synthesis has been applied to the synthesis of:

1. Thyrotropin Releasing Hormone (TRH)
2. Luteinizing Hormone-Follicle Stimulating Hormone Releasing Factor (LH-FSH-RF)
3. pyro-Glu-His-Try-$CONH_2$
4. pyruvyl-Tyr-Gly-Leu-Arg-Pro-Gly-$CONH_2$
5. pyro-Glu-His-Try-$CONH_2$ and pyruvyl-Tyr-Gly-Leu-Arg-Pro-Gly-$CONH_2$ from a single peptide chain using dehydroalanine in position 4 to generate the Try-$CONH_2$-function and the pyruvyl group
6. TRH (large scale for clinical purposes)
7. Melanotropin Release Inhibiting Hormone
8. Growth Hormone Releasing Factor Inhibiting Factor

Ten derivatives (different aminoacyl groups) of terto-butyl-oxycarbonyl dehydroalanine have been synthesized and characterized for use in the synthesis of peptide amides via dehydroalanine resins.

The conversion of dehydroalanine to amide and ketoacid is now applicable to the synthesis of amides in endo-positions. Model peptides with asparagine and/or glutamine in such positions have been synthesized with excellent yields.

Significance to Biomedical Research and the Program of the Institute:

All known hormone releasing factors and many other biologically important peptides carry the amide function in the COOH-terminal position, possibly for reasons of delayed degradation in the physiological environment. The application of α,ß-unsaturated amino acids to the generation of COOH-terminal amides and their utility as carriers for peptides on solid supports grew from the structural work with peptides with dehydroalanine and dehydrobytyrine (Nisin, Subtilin, etc.). The conversion of α,ß-unsaturated amino acids to the amides (and ketoacids) proceeds under mildly acidic conditions with the simultaneous release of the peptide from its support. Hormone releasing factors synthesized via this method display the full biological activity of the native counterpart and/or better biological properties than products derived via different synthetic routes. The method is flexible and adaptable to many needs of the synthesis of biologically active peptides, inclusive of the incorporation of essential amide functions in endo-positions and the multiple synthesis of peptide amides from single peptide chains. Certain hormone releasing factors are focal points of interest with regard to their potential in fertility regulation.

Proposed Course of Project:

(1) Synthesis of analogues of gramicidin A, B and C; (2) synthesis of radiolabeled gramicidins; (3) synthesis of nisin, fragments of nisin, and analogs of these peptides with and without radioactive amino acids; (4) synthesis of hormone-releasing factors of peptide structure; (5) synthesis of subtilin,

fragments of subtilin, and analogs of the parent molecule, and partial structures; (6) development and improvement of techniques of peptide synthesis; (7) extension of the principles of the solid phase synthesis of peptide amides via α, β-unsaturated amino acids to the protection of functional groups in amino acids.

Serial No. HD RPL-7
1. Reproduction Research Branch
2. Section on Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Cell Organelles from Human Placenta: Formation, Isolation, Characterization, and Function Under Normal and Abnormal Conditions

Previous Serial Number: HD-LB14

Principal Investigator: E. Gross

Other Investigators: W. Gerber

Cooperating Units: I. Boime, Washington University, School of Medicine, St. Louis, Missouri

Man Years:

Total: 1-1/2
Professional: 1-1/2
Others: 0

Project Description:

Objectives:

1. Isolation and characterization of cell organelles, primarily lysosomes,
2. Study of the lysosomal membrane: chemical composition, reactivity, permeability, structural enzymes;
3. Constituents of lysosomes: isolation and characterization of enzymes, basic proteins, and other components;
4. Correlation of lysosomal enzymes and their function with the life cycle of the cell under normal and pathological conditions;
5. Analysis of lysosomal enzyme levels in the human placenta at various stages of gestation and the role they may play in human fetal development;
6. Preparation of cell-free systems of protein synthesis using ribosomes isolated from first and third trimester human placental tissue.
7. Study of the effect of peptides with α, β-unsaturated amino acids on isolated lysosomes from various tissue sources.

Methods Employed:

Ultracentrifugation, zonal centrifugation, carrier-free electrophoresis, techniques of enzyme analysis, fluorescence and electron microscopy, separation techniques for biopolymers.

Major Findings:

Lysosomes exist in human term placental tissue, the syncytium being the prime source of the organelle. Fifty percent of the placental lysosomes are associated with the endoplasmic reticulum. The remainder displays the density properties found for lysosomes from other tissue sources. The lack of specificity makes acid phosphatase a poor marker enzyme for placental (and possibly) other lysosomes. The glucose 6-phosphatase hydrolyzing enzyme of human term placenta is not necessarily identical with glucose 6-phosphatase from other tissues. Peroxisomes are absent from human term placenta. Two populations of lysosomes with similar buoyant density profiles are present in first trimester human placental tissue. There is an increase in buoyant density with progressing gestation. First trimester human placental lysosomes display considerable heterogeneity with regard to density distribution and latency of certain acid hydrolases. First trimester placenta is richer in lysosmes than term placenta, a fact possibly related to postulated special functions in early gestation. The polysaccharide Stractan II (arabino galactan) is an excellent supporting medium for the age-dependent separation of erythrocytes. Stractan offers these advantages over previously used supporting media: simplicity of preparation, economy, readily adjustable osmolarity, low viscosity, and biologically inert behavior. Cell-free systems from first and/or third trimester human placenta support protein biosynthesis as directed by endogenous as well as exogenous messenger ribonucleic acids.

Significance to Biomedical Research and the Program of the Institute:

Lysosomes are of central significance throughout the life cycle of the cell. The large number of hydrolases present in the organelle perform numerous important functions under normal and abnormal conditions. Profound physiological events, such as those of reproduction, may depend upon the cells from different organs of the maternal as well as the fetal organism during development. The preparation of active lysosomes from first and third trimester human placental tissue shows that it will be possible throughout the entire course of pregnancy to study the endogenous synthesis of (a) placental proteins in general, (b) that of placental protein hormones in particular. Peak levels of certain hormones, for instance chorionic gonadotropin and lactogen occur at different gestational times. The investigation of their cell-free synthesis, liberation, and secretion will be most informative.

Proposed Course of Project:

(1) Improvement of techniques for the isolation of lysosomes from different types of developing tissue; (2) isolation and characterization of lysosomal enzymes and other protein constituents from the organelle; (3) elucidation of the structure and function of lysosomal membrane; (4) study of lysosomes under normal and abnormal conditions; (5) investigation of the effect of low molecular weight peptides and proteins on the lysosomal membrane; (6) correlation of the structure of membrane-active compounds with structural features of the lysosomal membrane; (7) investigation of the enzyme content of human placental lysosomes at various stages of gestation; (8) study of the cell-free protein synthesis of placental protein hormones; (9) extension of the advantages of using Stractan II as supporting medium to the isolation of subcellular constituents.

Serial No. HD-RPL-9
1. Reproduction Research Branch
2. Section on Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Chemical Modification of Amino Acids. Selective Nonenzymatic Cleavage of Peptide Bonds. Structure-Function Relationship of Biologically Active Peptides and Enzymes

Previous Serial Number: HD-LB-19

Principal Investigator: E. Gross

Other Investigators: J. L. Morell, J. Brown, E. Nebelin, H. C. Chen, S. Nanno

Cooperating Units: L. C. Craig, Rockefeller University, New York; E. D. Becker, Laboratory of Physical Biology, NIAMDD; E. Scoffone, University of Padova, Padova, Italy; Endocrine Laboratories of Madison, Madison, Wisconsin.

Man Years:

Total: 3
Professional: 3
Other: 0

Project Description:

Objectives:

1. Chemical modification of reactive groups in polyfunctional amino acids;
2. Selective cleavage of peptide bonds (nonenzymatic fragmentation) by application of chemical reagents;
3. Production, isolation, and purification of biologically active peptides and enzymes;
4. Fragmentation of biologically active peptides and enzymes with the objective of studying residual activity;
5. Synthesis of enzyme inhibitors and study of inhibitory effects, identification of the site of inhibition and of loci responsible for enzyme activity;
6. Isolation, purification, characterization, and elucidation of structure-function relationships of peptide and protein hormones, and hormone releasing factors.

Methods Employed:

(1) Enzymes; (2) chemical reagents for nonenzymatic fragmentation;

(3) high energy radiation techniques; (4) photochemical techniques; (5) advanced separation and analytical techniques: chromatography, electrophoresis, countercurrent and counter double current distribution, automatic sample application in the amino acid analysis, electronic equipment in data acquisition and processing; (6) nuclear magnetic resonance; (7) mass spectroscopy; (8) culturing techniques and bioassays; (9) dialysis, and (10) ultrafiltration techniques.

Major Findings:

(1) Nisin: The ß-carbon atoms of the ß-methyllanthionine residues in nisin possess L-configuration, i.e., the α-aminobutyric acid moieties of this amino acid with three centers of asymmetry are present in the threo-form. The synthesis of model peptides with dehydrobutyrine, their physicochemical investigation and comparison with dehydrobutyrine-containing fragments from nisin point to the Z-conformation for the dehydrobutyrine residue in nisin. (2) Subtilin: The addition of benzylmercaptan to the dehydrobutyrine residue in subtilin and/or fragments of subtilin yields products which support the presence of the Z-conformer also in subtilin. NMR-Data support this conformational assignment. Amino acid analytical and chromatographic studies establish the L-configuration for the ß-carbon atoms of the ß-methyllanthionine residues of subtilin, thus ascertaining identity between nisin and subtilin also in this respect. (3) The structural elucidation of cinnamycin and duramycin has advanced to the point that these partial structures may be written:

Cinnamycin:

$\mathrm{H_2N\text{-}ALA\text{-}ARG\text{-}GLU\text{-}ALA\text{-}ALA\text{-}PHE\text{-}(ABA_2ALA_1ASP_1\ HO\text{-}ASP_1PRO_1GLY_1}$

$\mathrm{PHE_2\ VAL_1)}$-lysinoalanine-COOH

Duramycin:

$\mathrm{H_2N\text{-}ALA\text{-}LYS\text{-}GLU\text{-}ALA\text{-}ABA\text{-}(ABA_1ALA_2ASP_1\ HO\text{-}ASP_1\ GLY_1,\ \text{-}PHE\text{-}GLY\text{-}}$

PRO-PHE-PHE-, VAL)-lysinoalanine-COOH

All residues shown as ALA and ABA are in sulfide bond to form lanthionine and/or ß-methyllanthionine.

(4) Lysinoalanine is formed upon alkaline treatment of nisin, subtilin, and the COOH-terminal fragment of nisin. The working hypothesis calls for the 1/2-addition of the epsilon amino group of lysine across the double bond of dehydroalanine during biosynthetic events. (5) The Chemical Synthesis of Nisin has been initiated with the preparation of the peptide ALA-S-ABA-PRO-GLY, a partial sequence of the tryptic fragment T_1. (6) Ultrafiltration techniques are replacing the earlier extraction procedures to improve the isolation of gonadotropins from monkey urine. (7) The purification of nisin on a large scale and its enzymatic fragmentation provides new and smaller fragments than previous ones for biological investigation, particularly the studies related to fetal resorption. (8) Glutamic acid, esterified and reduced to α-amino-delta-hydroxy valeric acid, offers a new approach to the nonenzymatic fragmentation of peptides and proteins. (9) The cleavage of the

aminoacyl bond of S-methyl-cysteine with cyanogen bromide proceeds to the extent of 80% in fragments of bovine pancreatic ribonuclease. (10) The low rate of cleavage of the methionyl-threonyl bond with cyanogen bromide is due to the participation of the ß-hydroxyl function of threonine in a competing reaction.

Significance to Biomedical Research and the Program of the Institute:

Knowledge of the details of the structure of peptides, such as nisin, subtilin and the gramicidins A, B, and C is a prerequisite for the understanding of the mode of action in their effects on intrauterine development. The working hypothesis, on the basis of which these peptides were entered into studies on fetal resorption, calls for the addition of essential sulfhydryl groups across the constituent α,ß-unsaturated amino acids. This reversible reaction is likely to be linked to other components of physiological significance, for instance amides and ketoacids. The former are the unique terminating element of peptides with hormone releasing properties. It remains to be seen, whether peptides with ketoacyl groups at H_2N-terminal residues are of importance in developmental physiology and/or fertility regulating processes.

Proposed Course of Project:

(1) Development and application of nonenzymatic methods to the structural elucidation of peptides and proteins: (a) application of selective oxidants to the cleavage of tryptophyl peptide bonds; (b) study of the selective suppression of the cleavage of methionyl peptide bonds upon treatment with cyanogen bromide; (c) fragmentation of reduced and S-alkylated peptides and proteins; fragmentation of peptides and proteins subsequent to mercaptan addition to α,ß-unsaturated amino acids; direct fragmentation at ,ß-unsaturated amino acids, at times preceded by ß-elimination; (d) the fragmentation at aspartic acid residues after esterification and reduction to be developed to a generally applicable method of nonenzymatic fragmentation.

(2) Nisin: Structural elucidation of nisin-like peptides from Streptococcus lactis. (3) Subtilin: Determination of the geometric isomerism of dehydrobutyrine. (4) Cinnamycin and Duramycin: Structural elucidation and structural correlation. (5) Nisin and Subtilin: Phylogenetic aspects of the structure of peptides with α,ß-unsaturated amino acids and lanthionines. (6) Preparation of peptides from natural sources and by chemical synthesis for screening purposes to establish inhibitory effects on fetal growth.

Serial No. HD-RPL-10
1. Reproduction Research Branch
2. Section on Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Physical Chemistry of Membrane-Active Peptides. Peptides with α,β-Unsaturated Amino Acids and Lanthionines and Their Effect on Neonatal and Neoplastic Tissue.

Principal Investigator: E. Gross

Cooperating Units: P.B. Chock, Laboratory of Chemistry, NHLI, Drug Development Branch, NCI.

Man Years:

Total: 1/2

Professional: 1/2

Project Description:

Objectives:

1. Structural and conformational analysis of peptides with α,β-unsaturated amino acids and lanthionines;
2. Structural and conformational analysis of peptides with other unusual amino acids, e.g., alternating configurational differences;
3. Analysis of the ion-binding and complexing properties of these peptides;
4. Analysis of the ion-transport properties of peptides with the characteristics indicated above;
5. Study of the mode of interaction of these peptides with constituents of membranes and biomembranes.

Methods Employed:

Spectral techniques: UV, IR, and spectroscopy in the visible range; Raman spectroscopy; nuclear magnetic resonance spectroscopy; X-ray analytical techniques; electron spin resonance measurements; atomic absorption analysis; bioassay techniques to establish biological parameters and their changes as brought about by the action of this class (and or classes) of peptides.

Major Findings:

NMR-Studies: The vinyl protons of N-acetyldehydroalanine are magnetically inequivalent in dimethyl-d_6-sulfoxide and aqueous solutions at deuterium concentrations other than those of pH 2.1. In deuterated solvents there is no exchange of deuterium with the vinyl protons of dehydroalanine indicating that N-acetyldehydroalanine is present predominantly in the enamine form.

Atomic absorption: Nisin and nisin fragments are capable of complexing alkali metal ions (na^{+}; K^{+}).

Bioassay data: Nisin, nisin fragments, and gramicidin A, B, C induce feta resorption in rabbits and rats.

Nisin, nisin fragments, and gramicidin A, B, C affect the growth of neoplastic tissue. T/C-values greater than 120.

Significance to Biomedical Research and the Program of the Institute:

Knowledge of subtle structural details is essential for the understanding of the mode of action of peptides on membranes and biomembranes. Truly minor deviations from necessary conformational requirements may make all the difference in the success or failure of the action of a peptide on a biomembrane. These molecular refinements are only determinable with the most sensitive physicochemical techiques, some of which are indicated above. Their understanding and control, however, are all-important for the elucidation of delicate regulatory mechanisms, not the least within the domain of developmental physiology.

Proposed Course of Project:

Nuclear Magnetic Resonance studies on nisin, nisin fragments, and the gramicidins A, B, and C. Broadly based spectral studies of the interactions of these peptides with cellular and subcellular constituents isolated from developing tissue, e.g., lysosomes from human placenta of different gestational age. Extension of these studies to synthetic peptides of the same structural characteristics, however, specifically labeled with various isotopes (tritium, carbon-13, sulfur-35).

Ion binding, complexing, and transport studies with the above peptides on synthetic membranes of known composition and on biomembranes of well-characterized origin.

Evaluation and correlation of biological effects as brought about by these peptides at the cellular and subcellular level.

Serial No. HD-RPL-12
1. Reproduction Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Structure and Function of Proteins

Principal Investigator: J. S. Cohen

Other Investigators: M. B. Hayes

Cooperating Units: R. Wasylishen, Laboratory of Chemical Physics, NIAMDD, D. Westmoreland and R. C. Matthews, Stanford University, H. Dagenmeier, University of Tubingen, Germany

Man Years:

Total: 3
Professional: 3
Other: 0

Project Description:

Objectives:

1. To obtain detailed information on the structure and function of proteins principally by the use of proton magnetic resonance, exploring the capability of resolving resonances of individual atoms within a protein molecule in solution by this technique.
2. To extend the application of the technique of high resolution NMR to proteins.

Major Findings:

Studies of the histidine C2 proton resonances of ribonuclease have continued to yield information on the environments of these residues in the protein. In particular, the resonance assigned to histidine 48 exhibits different titration curves in solutions of 0.1 M NaCl and 0.2 M Na acetate. Under the same conditions the curves of the other three resonances are essentially superimposable. The two curves represent two different environments for the side chain of histidine 48, and these probably explain the peculiar characteristics of this residue previously observed.

Titration data at different temperatures have been obtained for the histidine residues of ribonuclease (in collaboration with D. Westmoreland and R. Matthews, formerly of Stanford University). These have been analyzed to provide ionization constants and enthalpies and entropies of ionization. The result contrasts with those of an earlier study (Roberts et al. Biochem. 8: 2053, 1968). In particular, the inflection present in the titration curve of histidine 119, due to interaction with aspartic acid 121, gives a large negative entropy value, indicating considerable disordering of the solvent molecules around these groups upon protonation.

In previous studies on horse cytochrome two histidine C2 proton resonances were observed. One gave a complete titration curve; the other was seen at low pH. These resonances were monitored as part of a study of the acid denaturation of horse cytochrome and apocytochrome C (J. Biol. Chem. 249: 1113, 1974). It has been possible to assign these resonances to individual histidine residues in the protein by comparison with the homologous tuna cytochrome, in which the histidine residue position 33 is replaced by a tryptophan residue. This has allowed a more detailed analysis of the denaturation process previously described, utilizing the known X-ray structures of cytochromes.

Sperm whale and horse myoglobins contain 12 and 11 histidine residues respectively. In previous comparative studies of these myoglobins, we had observed 7 and 6 titrating histidine resonances respectively (Cohen et al. J. Mol. Biol. 71:513, 1972). In recent work we have obtained data on human myoglobin showing four titrating histidine resonances. Since this protein contains 9 histidines, there is a difference of 5 unobserved in each case. The simpler analysis in this case provides confidence in the continuities chosen in the earlier data and confirms the presence of a high-field, high pK titration curve. Differences in the curves enable assignments of individual curves to individual histidine residues. Data on the effect of azide ion on these titration curves and pH-titrations in the presence of excess azide are being analyzed. On the basis of earlier work it appears that the high pK titration curve is greatly affected by azide and may be histidine residue 64, "proximal" to the heme group.

The spin-lattice relaxation time (T_1) of a nucleus is a measure of its mobility. In order to provide a background to studies in proteins in this relatively new area, we studied the pH-dependence of the T values of the protons of imidazole in solution. A distinct minimum was observed around neutral pH, and following studies with EDTA and Cu^{2+}, this was attributed to the effects of paramagnetic metal ion at impurity levels down to 10^{-7} M. The sensitivity of this effect has important implications for such studies. Several T_1 values of individual histidine resonances in proteins measured by ourselves and others were interpreted in the light of these results. The work is now being extended to polyhistidine, particularly in view of the efficient nature of the Cu-polyhistidine complex in oxidative studies and of an extensive literature on the nature of such complexes. Other work involving spin-spin relaxation times (T_2) of resonances in proteins and a consideration of the important relationship of T_1 and T_2 are underway.

Proposed Course of Project:

Emphasis will be placed on the myoglobins and relaxation time studies.

Serial No. HD-RPL-13
1. Reproduction Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Application of Stable Isotopes to Biochemical Problems

Principal Investigator: J. S. Cohen

Cooperating Units: J. Chaiken, Laboratory of Chemical Biology, NIAMDD,
E. Sokoloski, Laboratory of Chemistry, NHLI.

Man Years:

Total: 0.2

Professional: 0.2

Others: 0

Objectives:

1. To develop the use of stable isotopes (^{13}C, ^{15}N) in biochemical systems with the application of appropriate techniques (NMR, GLC-MS);
2. To prepare, chemically or biosynthetically, molecules containing these isotopes for studies of structure and function.

Major Findings:

Histidine, carbon-13 (enriched (90%) at the C2 position in the imidazole ring, was incorporated by peptide synthesis into the (1-15) peptide of RNase. The microenvironment of this resonance was then studied in the peptide and the intact RNase-S' prepared by complexation with native RNase S-protein. This work complements earlier work using 15% C^{13}enriched amino acids incorporated in the (1-15) peptide.

Proposed Course of Project:

The isolation of amino acids from algae enriched to 80% in ^{13}C (gift, Los Alamos Scientific Labs.) is underway, following established procedures (J. Cohen and I. Putter, Biochim. Biophys. Acta, 222:515, 1970). Work on the NMR coupling constants and GLC-MS of these materials is projected (some will be supplied to LCS/NIMH for metabolic studies). Some amino acids will be incorporated synthetically into model peptides and peptide hormones (in collaboration with E. Gross) for ^{13}C NMR studies. Incorporation by cell culture systems will be considered.

Serial No. HD-RPL-14
1. Reproduction Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Binding Studies of Ligand or Hormones using NMR.

Principal Investigator: J. S. Cohen

Cooperating Units: P. Cohen and J. Griffin (CEN Sacloy, France), John Driscoll and Li-Ming Twan Moh, Detection and Diagnosis Branch, NCI and D. Cooney, Laboratory of Toxicology, NCI.

Man Years:

Total: 0.2

Professional: 0.2

Other: 0

Project Description:

Objectives:

1. To study the binding properties of ligands and hormones to protein sites, mainly utilizing the high resolution and dynamic characteristics of NMR.

Major Findings:

A study on the binding of the peptide hormones oxytocin and lysine vasopressin to neurophysins has been concluded. Major findings were that fast exchange of the peptides on the protein sites was demonstrated from the temperature dependence of the spin-spin relaxation time (T_2), in contrast to a report of slow exchange (Balaram et al., Biochem. 12:4695, 1973). A rotational correlation time of 5×10^{-8} sec for the tyrosine (and phenylalanine) residues at positions 2 (and 3 in LVP) were obtained indicating that these residues are markedly restricted upon complex formation, suggesting that they are directly involved in the binding with neurophysin. Differences in the effects for oxytocin and LVP complement other recently observed differences in the binding properties of these peptides.

The three resolved proton resonances of L-aspartic acid show selective effects on the addition of aliquots of asparaginase from two sources (Erwinia and E coli). Notably the ß CH_aH_b resonances are more affected than the $_\alpha$CH, indicating the ß carboxyl group is most restricted upon binding. The temperature dependence of T_2 indicates slow exchange.

Proposed Course of Project:

The aspartic acid-asparaginase preliminary observations will be extended, in relation to equilibrium dialysis and other data of Dr. Cooney's. Other systems of this type will be studied as appropriate.

Serial No. HD-RPL-2
1. Reproduction Research Branch
2. Section of Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Testicular Factors Regulating Secretion of Pituitary Gonadotropins (1973 Title - Physiology of Testis)

Principal Investigator: G. D. Hodgen

Other Investigators: W. E. Nixon, B. J. Gulyas, P. M. Krueger and W. W. Tullner

Man Years:

Total: 4
Professional: 3
Other: 1

Project Description:

Objectives:

1. To understand factors regulating FSH and LH secretion in males;
2. Construct suitable animal models to simulate conditions of infertility in man.

Major Findings:

Proceeding from last years observation that γ-glutamyl transpeptidase (GTP) is an enzyme marker for mature Sertoli cells in the rat, it has been shown in the germinal cell depleted vitamin A deficient rat that maintenance of normal Sertoli cells is associated with normal control FSH levels. These findings suggest that the seminiferous tubular factor regulating pituitary FSH secretion may come from within the Sertoli cell.

Castrated male rats were injected with tissue homogenate of adult or juvenile testes or epididymes. Serum LH levels were significantly reduced and that of FSH was slightly depressed by homogenates of adult testes. There was no effect of adult or juvenile epididymes or juvenile testes homogenates on either gonadotropin. Adult and juvenile testicular tissues contained approximately the same concentrations of testosterone. These findings indicate that a factor present in adult testes, probably not testosterone, is effective peripherally in suppressing gonadotropin secretion. Also, this factor is not associated with mature germinal cells or seminiferous tubular fluid which pass from the testis into the epididymis.

Our earlier studies identified three germ cell specific isozymes of lactic dehydrogenase (LHD-X) in addition to the ubiquitous LDH isozymes.

Polyacrylamide gel was used to separate these isozymes from rat testis at varying stages of growth and development. Statistical analysis of their "Ferguson Plots" expressed in terms of their retardation coefficients (K) and their Y- intercepts (Y) showed that the isozymes were separable because of differences in net charge rather than molecular size or weight (140,000). The finding that the LDH-X isozymes are charge isomers supports the concept that the mechanisms involved in their synthesis may be the same as those for the ubiquitous LDH isozymes, despite their origins in either somatic or gametic cells.

Groups of male rhesus monkeys with known dates of birth, were studied at appropriate intervals beginning at ages 4 yr., 3 yr., and 1 yr. Parameters included were: plasma testosterone determinatins; testis biopsies, for light and electron microscopy; growth records (gonadal dimensions, body length and weight) and perineal skin color, testicular position (inguinal or scrotal) and dentition. Spermatogenesis was in progress in all monkeys monkeys in the series started at 4 yr. and one of these provided a fertile mating one year later. Spermatogenesis was in progress at 3 yr. 8 m,o. in 2 of 3 animals in the 3 yr. group. One animal showed no signs of spermatogenesis at 3 yr. 10 mo. In the 1 yr. group, there was no evidence of spermatogenesis after 2 1/2 years. Spermatogenesis in the 3 animals in the older group (4 yr.) preceded the attainment of adult levels of testosterone by 4 months. In the 3 yr. old series, the 2 animals with spermatogenesis at 3 yr. 8 mo. progressed to testosterone levels usually found in the lower range for adults at 4 yr. 1 mo. Testosterone levels in the most immature group (1 yr.) remained at low levels throughout the 15 month period of study. It It is clear that adult levels of testosterone are not a prerequisite to the initiation of spermatogenesis. Other aspects of this investigation are still in progress.

Proposed Course of the Project:

Complete the ultrastructural studies and compile data on the development of the testes of rhesus monkey during fetal period, postnatal to onset of puberty, and puberty through adulthood.

Investigate the effects of various nutritional factors on concentrations of serum and pituitary gonadotropins in rats.

Complete development of radioimmunoassays for FSH and LH in rhesus monkeys and use these assays to examine gonadal factors regulating FSH and LH secretion in this primate.

Serial No. HD-RPL-3
1. Reproduction Research Branch
2. Section on Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Fertilization and Embryogenesis

Principal Investigator: G. D. Hodgen

Other Investigators: B. J. Gulyas

Man Years:

Total: 1 1/2
Professional: 1
Other: 1/2

Project Description:

Major Findings:

In the rabbit, upon fertilization of the ova, the zona pellucida is rendered resistant to proteolysis. It is believed that this change in the zona pellucida is brought about by the content of the cortical granules which are released at fertilization. In the rabbit, block to polyspermy is accomplished by the plasma membrane concomitant with the increased resistance of the zona pellucida to proteolysis. When rabbit oocytes are parthenogenically activated 60% of them form pronucleus or pronuclei. These ova fail to release their cortical granule content into the perivitelline space and their zona pellucida behave similar to those of unfertilized oocytes when subjected to proteolysis. Presently unfertilized oocytes are being electrically stimulated in an effort to release the cortical granules and activate them parthenogenically. The zona pellucida of these ova will be subjected to proteolysis. Finally, we demonstrated that the addition of bicarbonate during proteolysis will accelerate the lysis of the zona pelucida of unfertilized eggs and facilitates removal of the zona pellucida of fertilized eggs within 10 minutes, a task that could not be accomplished previously.

Proposed Course of the Project:

Continue the presently ongoing project on the release of cortical granule content of rabbit oocytes and its relevance to block to polyspermy and the increased resistance of the zona pellucida to proteolysis. Attention will be focused on the role of bicarbonate during proteolysis of the zona pellucida of fertilized eggs.

Serial No. HD-RPL-4
1. Reproduction Research Branch
2. Section on Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Hormonal Interrelationships Between Fetus, Mother and Placenta Throughout Gestation in Rhesus Monkeys (1973 Title - The Use of the Rhesus Monkey in Studies of Pregnancy)

Principal Investigator; G. D. Hodgen

Other Investigators: W. W. Tullner, B. J. Gulyas, and G. T. Ross

Man Years:

Total: 4
Professional: 3
Other: 1

Project Description:

Objectives:

1. To understand the hormonal interrelationships between maternal and fetal endocrine tissues as well as the placenta;
2. To describe the intrauterine hormonal dependencies of the fetal gonads and adrenals;
3. To assess separately the contribution of the placenta to the hormonal milieu present during gestation;
4. To assess the functional status of the maternal and fetal pituitaries during gestation.

Major Findings:

In order to study the role of the fetus and ovaries in the production of steroids by pregnant rhesus monkeys, concentrations of estrogens and progesterone were measured in peripheral plasma of fetectomized or ovariectomized monkeys and compared with those of intact, pregnant females. Within three weeks after fetectomy, estradiol levels had fallen to one-third that of controls; whereas, progesterone levels were unaffected by removal of the fetus. In addition, the placentas were retained in a viable state until placentectomy near the time of expected parturition. Except for a brief nadir in estrogen levels immediately following early ovariectomy, removal of the ovaries had no demonstrable influence on circulating levels of estrogens or progesterone. The data indicate that the fetus makes the major contribution to the estradiol found in maternal serum. Lastly, it is the placenta, not the fetus or corpus luteum, which sustains progesterone secretion in advanced gestation.

The corpus luteum of pregnancy in the rhesus monkey regresses into the ovary during mid-pregnancy. It was demonstrated that three to four weeks prior to the expected day of parturition the corpus luteum of pregnancy (CLP) emerges to the surface of the ovary and protrudes from it. Simultaneously the CLP becomes vascular. Light microscopic observations indicate that the granulosa lutein cells have the histological appearance suggesting secretory function.

The granulosa lutein cells of the corpus luteum from rhesus monkeys at term pregnancy possess all the ultrastructural features essential for steroidogenesis. These observations are consistent with observations of others who considered the corpus luteum of pregnancy just prior to parturition functional, judging by the elevated ovarian venous progesterone output. Furthermore, using electron microscopic histochemical techniques, we found that the granulosa lutein cells are abundant in peroxisomes, small granules which are bounded by a single membrane and have homogeneous particulate content. Peroxisomes may contain various enzymes, but mostly oxidative enzymes which generate H_2O_2, and catalase, an enzyme that breaks down H_2O_2. Some enzymes of steroidogenesis are located in the microsomal fractions of the endoplasmic reticulum. It is possible that, just as in the adrenal cortex, the peroxisomes contain steroid dehydrogenases that are involved in cholesterol and steroid hormone synthesis. Ours is the first demonstration of peroxisomes in corpus luteum of any animal. It is most interesting that very recently an increased activity of Δ-3ß hydroxysteroid dehydrogenase has been demonstrated by biochemical means in the corpus luteum of rhesus, just prior to parturition. Thus our demonstration of peroxisomes in the corpus luteum at term pregnancy correlates with the increased venous progesterone output and increased dehydrogenase activity demonstrated by others.

The effects of maternal hypophysectomy on endocrine gland morphology and plasma hormone levels were studied in a series of pregnant monkeys (2-2 1/2 months gestation). Cortisol concentrations were examined in plasma from both mother and fetus or newborn. In normal monkeys, at parturition, the ratio of maternal to fetal plasma cortisol was from 2 to 3 to 1. However, in these hypophysectomized mothers, the ratios were reversed. The higher fetal cortisol levels were associated with a significant increase in fetal adrenal size and weight (normal: 88, hypophysectomized: 247 mg/100 gm body wt.). Hypertrophy of the fetal adrenals from hypophysectomized mothers was due to a marked increase in the width of the zona reticularis as well as an increase in cell size in the other two cortical zones. The maternal adrenal cortex was characterized by a marked atrophy of the zona fasiculata and zona glomerulosa. Histological assessment indicated that one of five animals in this series was incompletely hypophysectomized. It is known that a bidirectional transfer of cortisol occurs between mother and fetus in this primate although placental conversion of much of the maternal cortisol to cortisone results in a less active metabolite entering the fetal compartment. Present findings indicate that lack of normal maternal cortisol secretion is associated with compensatory hypertrophy of the fetal adrenal cortex and a significantly increased fetal cortisol production. In addition, when the fetuses were hypophysectomized at 115 days in gestation, the term fetal adrenal, gonadal and thyroid weights were 1/15, 1/2 and 1/2 that of their respective intact, control fetuses--suggesting that maternal pituitary tropic hormones may not cross over into the fetal circulation.

Proposed Course of the Project:

Present studies will be extended by determining the dependency of fetal gonadal development on the hormonal milieu of the gestational process. This will involve coordination of both hormonal patterns and morphological changes associated with intra-uterine growth and development of this primate.

Hypophysectomized rhesus monkeys will be used to investigate the effects of estrogen sensitization on follicular responses to highly purified gonadotropins.

Serial No. HD-RPL-15
1. Reproduction Research Branch
2. Section of Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Measurement of Chorionic Gonadotropin in the Rhesus Monkey and Its Significance in Early Pregnancy

Principal Investigators: G. D. Hodgen

Other Investigators: G. T. Ross, J. L. Vaitukaitis, W. E. Nixon and W. W. Tullner

Man Years:

Total: 3
Professional: 2
Other: 1

Project Description:

Objectives:

1. To develop a specific radioimmunoassay for macaque chorionic gonadotropin (mCG);
2. To understand the role of mCG in the fertile menstrual cycle;
3. To develop a simple and rapid test system for the early diagnosis of pregnancy in sub-human primates.

Major Findings:

An antiserum to the β subunit of ovine LH has lead to two major accomplishments in the measurement of macaque chorionic gonadotropin (mCG).

The first is the development of a specific radioimmunoassay (RIA) for mCG that is 50 times more sensitive than the bioassays currently used. This mCG RIA has been used to demonstrate that the attaching blastocyst or the sycytiotrophoblast either already possesses or rapidly attains the capacity to secrete mCG. Further, the presence of mCG at the time of implantation probably maintains the viability of the corpus luteum and temporarily enhances progesterone secretion.

The second use of this antiserum to oLHß has been the development of a hemagglutination inhibition test for urinary chorionic gonadotropin of rhesus monkeys. The test permits a reliable diagnosis of pregnancy by the 18th day after fertilization, requires 2 hours, costs less than $1.00 per test and yields a definitive result nearly two weeks earlier than present techniques of pregnancy diagnosis. In conjunction with the Contraceptive

Development Branch, Center for Population Research, these reagents will be made available for distribution to all biomedical scientists where the diagnosing and monitoring of early pregnancy in sub-human primates is important (including macaques, baboons, and higher apes).

Proposed Course of the Project:

Studies are planned to determine if the pre-implantation blastocyst synthesizes chorionic gonadotropin *in vivo* or *in vitro*.

Collaborative studies are in progress to determine if the mCG hemagglutination inhibition test will permit pregnancy diagnosis in lower primates, including marmosets and squirrel monkeys.

Methodology to extend the purification of rhesus chorionic gonadotropin (mCG) will be examined. The use of anti-ovine- LH-ß serum in affinity chromatography for this purpose is anticipated.

Honors and Awards:

Mortimer B. Lipsett

Elected Secretary-Treasurer Endocrine Society, 1974.

Invited Lectures:

University Teheran Reproductive Biology Unit, Teheran, October 1973
Pahlevi University School of Medicine, Sheraz, October 1973
Endocrine Society Postgraduate Course, Los Angeles, October 1973
New York University College of Medicine, New York, January 1974
University of Miami School of Medicine, Miami, April 1974
University of Minnesota School of Medicine, San Francisco, June 1974

Griff T. Ross

Elected, Association of American Physicians, 1974
Appointed Chairman of Medical Board, Clinical Center, NIH

Invited Lectures:

Chairman, Session at Laurentian Hormone Conference, Mt. Tremblant, August 1973
Endocrinology Symposium, Jacksonville Hospital, Jacksonville, September 1973
Department of Physiology, Medical College of Virginia, Richmond, February 1974
Visiting Professor, Department of Medicine, Baylor College of Medicine, Houston, March 1974

K. J. Catt

Invited Lectures:

Department of Physiology, Case Western Reserve University, Cleveland, September 1973
Rockefeller University, New York, December 1973
Department of Physiology, University of Michigan, Ann Arbor, January 1974
First Annual Workshop on the Testis, Houston, February 1974
Department of Medicine, Mt. Sinai Hospital, New York, February 1974
Laboratory of Human Reproduction, Harvard University, Boston, March 1974
Department of Animal Science, Cornell University, Ithaca, March 1974
Cleveland Clinic, Cleveland, April 1974
AFCR Symposium, Atlantic City, May 1974

E. Gross

Invited Lectures:

9th International Congress of Biochemistry, Stockholm, July 1973

Advanced Course No. 25, FEBS, Prague, July 1973
Department of Chemistry, Purdue University, April 1974
International Symposium on Isozymes, Yale University, April 1974
European Peptide Symposium, Jerusalem, May 1974

M. L. Dufau Catt

Elected American Society of Clinical Investigation, 1974

Invited Lectures:

First Annual Workshop on the Testis, Baylor University, Houston, 1974

A. Chrambach

Invited Lectures:

Tutorial Course on Quantitative PAGE, Biochemistry Department, University of Lund, Sweden, 1973
Max Planck Institute for Brain Research, Frankfurt, July 1973
FAES, Tutorial Course on PAGE, February 1974
Max Planck Institute for Brain Research, Frankfurt, March 1974

G. D. Hodgen

Invited Lectures:

Satellite Symposium of 6th Annual Meeting, Society for the Study of Reproduction, Athens, August 1973
Animal Reproduction Teaching and Research Center, Ohio State University, Columbus, September 1973

H. B. Pollard

Invited Lectures:

Department of Biochemistry, University of Chicago, Chicago, October 1973
Department of Neurology, University of California San Diego, November 1973
Department of Pharmacology, Stanford University, Palo Alto, November 1973
Department of Psychiatry, Stanford University, Palo Alto, November 1973
Brook Lodge Conference, Kalamazoo, June 1974

D. Rodbard

Invited Lectures:

Gordon Research Conference, Tilton, July 1973
International Atomic Energy Agency, Istanbul, September 1973
Chulalongkorn Hospital, WHO Research Unit, Bangkok, September 1973
Tokyo Women's Medical College, Tokyo, September 1973
University of Tokyo, Tokyo, September 1973
Japan Radioimmunoassay Society, Tokyo, September 1973
Duke University, Durham, October 1973

New York Medical College, New York, December 1973
University of Minnesota, Minneapolis, February 1974
Hormel Institute, Austin, February 1974
FAES, Tutorial Course on PAGE, February 1974

R. J. Sherins

Invited Lectures:

Serono International Symposium on Male Infertility, Milan, September 1973
Beth Israel Hospital, Boston, December 1973
Peter Bent Brigham Hospital, Boston, December 1973
Grand Rounds, Harvard Medical School, Boston, December 1973
Kimbraugh Seminar, Walter Reed Medical Center, Washington, D.C., January 1974
VII Post Graduate Course, American Fertility Society, Hollywood, April 1974

J. L. Vaitukaitis

Editorial Board, Journal of Clinical Endocrinology and Metabolism

Invited Lectures:

Rockefeller University, New York, November 1973
Memorial Hospital for Cancer and Allied Diseases, New York, November 1973
Applied Seminar on the Laboratory Diagnosis of Cancer, Association of Clinical Scientists, Washington, D.C., November 1973
Novak Memorial Lecture, Johns Hopkins School of Medicine, Baltimore, November 1973
Division of Veterinary Medicine, WRAIR, Washington, January 1974
UCLA School of Medicine, San Francisco, January 1974
University of Connecticut School of Medicine, Farmington, February 1974
Beth Israel Hospital, Boston, March 1974
Post Graduate Radioimmunoassay Course, Endocrine Society, Mayo Clinic, Rochester, March 1974
Post Graduate Course, Johns Hopkins University School of Medicine, Baltimore, May 1974
AFCR Symposium, Atlantic City, May 1974

PUBLICATIONS

Aloj, S. M., Edelhoch, H. A., Ingham, L. C., Morgan, F. J., Canfield, R. E., and Ross, G. T.: The rates of dissociation and reassociation of the subunits of human chorionic gonadotropin. Archives of Biochem. and Biophys. 159: 497-504, 1973.

Bardin, C. W., Bullock, L. P., Sherins, R. J., Mowszowics, I., and Blackburn, W. R.: Androgen metabolism and mechanism of action in male pseudohermaphroditism: A study of testicular feminization. Recent Prog. Horm. Res. 29: 65-109, 1973.

Braunstein, G. D., Vogel, C. L., Vaitukaitis, J. L., and Ross, G. T.: Ectopic production of hCG in Ugandan patients with hepatocellular carcinoma. Cancer 32: 223-226, 1973.

Braunstein, G. D., Whitaker, J. N., and Kohler, P. O.: Cerebellar dysfunction in Hand-Schuller-Christian Disease. Arch. Intern. Med. 132: 387-390, 1973.

Catt, K. J., and Baukal, A.: Prolonged retention of high specific activity by I-labeled II - consequence of 'decay catastrophe'. Biochimica et Biophysica Acta 313: 221-225, 1973.

Catt, K. J. and Dufau, M. L.: Spare gonadotropin receptors in the rat testis. Nature New Biology 244: 219, 1973.

Chrambach, A., Doerr, P., Finlayson, G. R., Miles, L.E.M., Sherins, R. J., and Rodbard, D.: Instability of pH gradients formed by isoelectric focusing in polyacrylamide gel. Ann. N.Y. Acad. Sci. 209: 44-64, 1973.

Chrambach, A., Yadley, R. A., Ben-David, M., and Rodbard, D.: Isohormones of human growth hormone. I Characterization by electrophoresis and isoelectric focusing in polyacrylamide gel. Endocrinology 93: 848-857, 1973.

Corash, L., and Gross, E.: Subcellular constituents of humam placenta. Isolation and characterization of lysosomes from term tissue. Pediat. Res. 7: 798, 1973.

Corvol, P., Rodbard, D., Drouet, P., Catt, K. and Menard, J.: Monoiodinated angiotensins: Preparation and characterization by polyacrylamide gel electrophoresis and isoelectric focusing. Biochem. Biophys. Acta 313: 221-225, 1973.

DeVita, V. T., Jr., Arseneau, J. C., Sherins, R. J., Canellos, G. P. and Young, R. C.: Intensive chemotherapy for Hodgkins Disease: Long term complications. J. Nat. Cancer Institute Monograph 36: 447-454, 1973.

Dufau, M. L., Charreau, E. H., and Catt, K. J.: Characteristics of a soluble gonadotropin receptor from the rat testis. J. Biol. Chem. 248: 6973-6982, 1973.

Dufau, M. L., Charreau, E. H., Ryan, D. and Catt, K. J.: Soluble gonadotropin receptors of the rat ovary. F.E.B.S. Letters 39: 149, 1974.

Dufau, M. L., Ryan, D. and Catt, K. J.: Disulphide groups of gonadotropin receptors are essential for specific binding of hCG. Biochim. Biophys. Acta 343/2: 417, 1974.

Glossman, H., Baukal, A. J., and Catt, K. J.: Angiotensin receptors in bovine adrenal cortex: Modification of angiotensin II binding by guanyl nucelotides. J. Biol. Chem. 249: 664, 1974.

Glossman, H., Baukal, A. J. and Catt, K. J.: Properties of angiotensin II receptors in the bovine and rat adrenal cortex. J. Biol. Chem. 249: 825, 1974.

Goldenberg, R. L., Grodin, J. M., Rodbard, D., and Ross, G. T.: Gonadotropins in women with amenorrhea. Am. J. Obstet. Gynecol. 116: 1003-1012, 1973.

Goldenberg, R. L., Reiter, E. O., Vaitukaitis, J. L., and Ross, G. T.: Hormonal factors influencing ovarian uptake of human chorionic gonadotropin. Endocrinology 92: 1565-1567, 1973.

Gross, E., Kiltz, H. H., and Craig, L. C.: The amino acid composition of subtilin. Hoppe Seyler's Z. Physiol. Chem. 354: 799, 1973.

Gross, E., Kiltz, H. H., and Nebelin, E.: The structure of subtilin. Hoppe Seyler's Z. Physiol. Chem. 354: 810, 1973.

Gross, E., Noda, K., and Nisula, B.: Solid phase synthesis via , -unsaturated amino acids. I. Peptides with carboxl-terminal amino amides - thyrotropin releasing factor (TRF) Angew. Chem. 85: 672, 1973; Angew. Chem. Internat. Edit. 12: 664, 1973.

Gulyas, B. J.: Cytokinesis in the rabbit zygote: Fine-structural study of the contractile ring and the mid-body. Anat. Rec. 177: 195-208, 1973.

Gulyas, B. J.: The corpus luteum of the rhesus monkey (Macaca mulatta) during late pregnancy. An electronmicroscopic study. Amer. J. Anat. 139: 95-122, 1974.

Hodgen, G. D., Nixon, W. E., and Turner, C. K.: Suppression of serum gonadotropins in castrated rats by testis homogenates. Intern. Res. Comm., Endocrine System 2: 1233, 1974.

Hodgen, G. D., Nixon, W. E., Vaitukaitis, J. L., Tullner, W. W., and Ross, G. T.: Neutralization of primate chorionic gonadotropin activities by antisera against the subunits of human chorionic gonadotropin in radioimmunoassay and bioassay. Endocrinology 92: 705-709, 1973.

Hodgen, G. D., and Sherins, R. J.: Enzymes as markers of testicular growth and development in the rat. Endocrinology 93: 985-989, 1973.

Kleinman, L. M., Tangrea, J. A., Gallelli, J. F., Brown, J. H., and Gross, E.: Assay and stability of a solution of essential amino acids. Am. J. Hosp. Pharm. 30: 1054, 1973.

Kiltz, H. H. and Gross, E.: Subtilin III. Enzymatic fragmentation with trypsin and thermolysin. Hoppe Seyler's Z. Physiol. Chem. 354: 802, 1973.

Kiltz, H. H. and Gross, E.: Subtilin IV. Sequence and assignment of sulfide bridges in the heterodetic bicyclic peptide of amino acid residues 20-29. Hoppe Seyler's Z. Physiol. Chem. 354: 805, 1973.

Lipsett, M. B.: The differential diagnosis of hursuitism and virilism. Arch. Intern. Med. 132: 616-619, 1973.

Lipsett, M. B.: Reproductive function in the human male. WHO Technical Report Series No. 520, Geneva, 1973, 34 pp.

McIlwaine, I., Rodbard, D., and Chrambach, A.: Characterization by polyacrlaymide gel electrophoresis of hemoglobins in rabbit embryo, fetus, and adult. Anal. Biochem. 55: 521-538, 1973.

Mecklenburg, R. S. and Lipsett, M. B.: Disappearance of metastatic breast cancer after oophorectomy. N. Engl. J. Med. 289: 845-846, 1973.

Mecklenburg, R. S., Noda, K., Miyachi, Y., Gross, E., and Lipsett, M. B.: LH-releasing activity of p-Glu-His-Trp-NH and p-Glu-His-Trp. Endocrinology 93: 993-997, 1973.

Menard, J. and Catt, K. J.: Effect of estrogen treatment of plasma renin parameters in the rat. Endocrinology 92: 813, 1973.

Miyachi, Y., Chrambach, A., Mecklenburg, R. S. and Lipsett, M. B.: Preparation and properties of ^{125}I-LHRH. Proc. of the Serono Foundation Conf. Acapulco, E. Rosemberg (Ed.) Excerpta Medica, Amsterdam 1972, pp. 92-97.

Miyachi, Y., Mecklenburg, R. S., Hansen, J. W., and Lipsett, M.B.: Metabolism of ^{125}I-luteinizing hormone-releasing hormone. J. Clin. Endocrinol. Metab. 37: 63-67, 1973.

Miyachi, Y., Mecklenburg, R. S. and Lipsett, M. B.: In vitro studies of pituitary-median eminence unit. Endocrinology 93: 492-496, 1973.

Morell, J. L., and Gross, E.: Configuration of the β-carbon atoms of the β-methyllanthionine residues in nisin. J. Am. Chem. Soc. 95: 6480-6481, 1973.

Mroczek, W. J., Finnerty, F. A. and Catt, K. J.: Lack of association between plasma-renin and history of heart attack or stroke in patients with essential hypertension. Lancet 2: 464, 1973.

Nebelin, E. and Gross, E.: Subtilin V. Sequence and assignment of sulfide bridges in the heterodetetic tricyclic peptide of amino acid residues 3-19. Hoppe Seyler's Z. Physiol. Chem. 354: 807, 1973.

Nisula, B. C., Kohler, P. O., Vaitukaitis, J. L., Hershman, J. M., and Ross, G. T.: Neutralization of human thyrotropin by antisera to subunits of glycoprotein. J. Clin. Endocrinol. Metab. 37: 664-669, 1973.

Nisula, B. C., Loriaux, D. L., Sherins, R. J., and Kulin, H. E.: Benign bilateral testicular enlargement. J. Clin. Endocrinol. 38: 440-445, 1974.

Pollard, H., Chace, N. M. and Coyle, J. R.: Dopamine-beta-hydroxylase from adrenal chromaffin granules: Evidence for a common glycoprotein subunit structure for soluble and membrane-bound forms. Brain Information Service 1: 35, 1973.

Pollard, H., and Chen, R.: Fluroescence and circular-dichroism studies of low density lipoprotein particles and lipid-depleted derivatives. J.

Pollard, H. B., Miller, A., and Cox, G. C.: Synaptic vesicles: Structure of chromaffin granule membranes. Second Annual Membrane Conference at Squaw Valley. J. Supramolecular Structure 1: 295-306, 1973.

Pollard, H., and Steers, E.: Beta-galactosidase from Bacillus megaterium, KM1: Isolation by affinity chromatography and characterization of the active species. Archives of Biochem. and Biophys. 158: 650, 1973.

Rodbard, D.: Toward a unified electrophoretic viewpoint. Ann. N.Y. Acad. Sci. 209: 515-529, 1973.

Rodbard, D.: Apparent positive cooperative effects in cyclic AMP and corticosterone production by isolated adrenal cells in response to ACTH analogues. Endocrinology, May 1974.

Rodbard, D. and Frazier, G. R.: Radioimmunoassay Data Processing; Second Edition. National Technical Information Service, Springfield, Va., USA 22151. (#PB 217366 for magnetic tape; #PB 217367 for printed listings), 1973, 224 pp.

Ruder, H. J., Loriaux, D. L., Sherins, R. J. and Lipsett, M. B.: Leydig cell function in men with disorders of spermatogenesis. J. Clin. Endocrinol. 38: 244-247, 1974.

Sherins, R. J. and DeVita, V. J., Jr.: Effect of drug treatment for lymphoma on male reproductive capacity. Ann. Int. Med. 79: 216-220, 1973.

Sherins, R. J. and Loriaux, D. L.: Studies on the role of sex steroids in the feedback control of FSH concentrations in men. J. Clin. Endocrinol. 36: 886-893, 1973.

Vaitukaitis, J. L.: Immunologic and physical characterization of human chorionic gonadotropin secreted by tumors. J. Clin. Endocrinol. Metab. 37: 505-514, 1973.

Vaitukaitis, J. L. and Ross, G. T.: Evaluation of recent advances in human gonadotropic hormones. Ann. Rev. Med. 24: 295, 1973.

Vaitukaitis, J. L., Ross, G. T., Pierce, J. G., Cornell, J. S., and Reichert L. E., Jr.: Generation of specific antisera with the hormone specific subunit of hTSH or hFSH. J. Clin. Endocrinol. Metab. 37: 653-659, 1973.

Yadley, R. A. and Chrambach, A.: Isohormones of human growth hormone. II. Plasmin-catalyzed transformation and increase in prolactin biological activity. *Endocrinology* 93: 858-865, 1973.

Yadley, R. A., Rodbard, D., and Chrambach, A.: Isohormones of human growth hormone. III. Isolation by preparative polyacrylamide gel electrophoresis and characterization. *Endocrinology* 93: 866-873, 1973.

REVIEWS AND CHAPTERS

Catt, K. J., Measurement of Hormones - Radioimmunoassay. In Berson, S. A. and Yalow, R. S. (eds.): *Methods in Investigative and Diagnostic Endocrinology, Vol. 2A, Peptide Hormones*, The Netherlands, North Holland Publishing Co., 1973, pp. 129-135.

Catt, K. J., Baukal, A. J. and Ashburn, M. J.: The renin-angiotensin system during oral contraceptive therapy and pregnancy. In M. Fregley (ed.): *Oral Contraceptives and High Blood Pressure*, Gainesville, Dolphin Press, 1974.

Catt, K. J., Baukal, A. J. and Ashburn, M. J.: Radioimmunoassay determination of plasma renin parameters and circulating angiotensin II. In Fregley, M. (ed.): *Oral Contraceptives and High Blood Pressure* , Gainesville, Dolphin Press, 1974.

Catt, K. J. and Dufau, M. L.: Interactions of LH and hCG with testicular gonadotropin receptors. In O'Malley, B. W. and Means, A. R. (eds.): *Receptors for Reproductive Hormones*, New York, Plenum Publishing Co., 1973, pp. 379-418.

Catt, K. J., Glossman, G., and Baukal, A. J.: Angiotensin II receptors of the adrenal cortex. In Sambhi, M. P. (ed.): *Mechanisms of Hypertension*, Amsterdam, Excerpta Medica, 1973, p. 200.

Dufau, M. L.: Bestimmung von Ostradiol 17 in Plasma durch Kompetitive Protein binding. In Breur, A., Kruskempe, H. L. and Hamel, D. (eds.): *Methoden der Hormonenbestimmung*. Stutgart, Georg. Thieme Verlag, 1974, p. 675.

Fontana, A. and Gross, E.: Solid phase synthesis of the pentadecapeptide valine-gramicidin A. In Hanson, H., and Jakubke, H. D. (eds.): *Peptides 1972*, Proceedings of the Twelfth European Peptide Symposium, Reinhardsbrunn, Germany, September 1972, Amsterdam, North Holland Publishing Co., 1973, p. 229.

Kapadia, G., Chrambach, A. and Rodbard, D.: Approaches to macromolecular mapping by polyacrylamide gel electrophoresis. In Allen, R. C. and Maurer, H. R. (eds.): *Electrophoresis and Isoelectric Focusing on Polyacrylamide Gel*, Berlin, Walter de Gruyter, Publ., 1974, p. 210.

Kohler, P. O. and Bridson, W. E.: Enhancement of growth hormone production by cortisol. In Kruse, P. G., Jr., and Patterson, M. K., Jr. (eds.): Tissue Culture Methods and Applications, New York, Academic Press, Inc., 1973, pp. 570-576.

Kohler, P. O. and Ross, G. T. (Eds.): Diagnosis and Treatment of Pituitary Tumors, Excerpta Medica, Amsterdam, The Netherlands, 1973.

Lipsett, M. B.: Hormonal induction of breast cancer. In Griem, M. L., Jensen, E. V., Ultmann, J. E. and Wissler, R. W. (eds.): Recent Results in Cancer Research, Vol. 42, New York, Springer-Verlag, 1973, pp. 28-30.

Lipsett, M. B.: Endocrine responsive cancers of man. In Williams, H. (ed.): Textbook of Endocrinology, Philadelphia, W. B. Saunders and Company, 1973, pp. 1071-1081.

Lipsett, M. B. and Sherins, R. J.: The Testis. In Bondy, Philip K. (ed.): Duncan's Diseases of Metabolism, Endocrinology, Seventh Edition, Philadelphia, W. B. Saunders, Co., 1974, pp. 1553-1584.

Mroczek, W. J., Finnerty, F. A. and Catt, K. J.: Plasma renin activity, myocardial infarction and cerebrovascular accidents in urban black hypertensive patients. In Sambhi, M. P. (ed.): Mechanisms of Hypertension, Amsterdam, Excerpta Medica, 1973.

Rodbard, D.: Mathematics of hormone-receptor interaction. I. Basic Principles. In O'Malley, B. W., and Means, A. R. (eds.): Receptors for Reproductive Hormones, New York, Plenum Publishing Co., 1973, pp. 289-326.

Rodbard, D. and Bertino, R. E.: Theory of radioimmunoassays and hormone-receptor interactions. II. Simulation of antibody divalency, cooperativity and allosteric effects. In O'Malley, B. W. and Means, A. R. (eds.): Receptors for Reproductive Hormones, New York, Plenum Publishing Co., 1973, pp. 327-341.

Rodbard, D.: Theory of hormone-receptor interaction. III. The endocrine target cell as a quantal response unit: A general control mechanism. In O'Malley, B. W., and Means, A. R. (eds.): Receptors for Reproductive Hormones, New York, Plenum Publishing Co., 1973, pp. 342-364

Rodbard, D. and Chrambach, A.: Quantitative polyacrylamide gel electrophoresis (PAGE): Mathematical and statistical analysis of PAGE data. In Allen, R. C. and Maurer, H. R. (eds.): Electrophoresis and Isoelectric Focusing on Polyacrylamide Gel, Berlin, Walter de Gruyter, Publ., 1974, p. 28.

Rodbard, D., Chrambach, and and Weiss, G. H.: Optimization of resolution in analytical and preparative polyacrylamide gel electrophoresis. In Allen, R. C. and Maurer, H. R. (eds.): Electrophoresis and Isoelectric Focusing on Polyacrylamide Gel, Berlin, Walter de Gruyter, Publ., 1974, p. 62.

Ross, G. T.: Pituitary-gonadal interactions in man before and after puberty. In Segal, S. J., Crozier, R., Corfman, P. A. and Condliffe, P., (eds.): *Regulation of Mammalian Reproduction*, Springfield, C. C. Thomas Co., 1972, p. 56-64.

Ross, G. T. and Vande Wiele, R. L.: The Ovaries. In Williams, R. H. (ed.): *Textbook of Endocrinology*, Philadelphia, W. B. Saunders, Co. 1973, pp. 368-422.

Sherins, R. J.: The evaluation and management of the infertile male. Annual Meetings of the American Fertility Society, 7th Post Graduate Course, New York, Februrary 27, 1972.

Sherins, R. J.: The evaluation and management of the infertile male. Syllabus 7th Post Graduate Course, Annual Meeting of the American Fertility Society, Hollywood, Florida, April 3, 1974.

Tullner, W. W.: Comparative aspects of primate chorionic gonadotropins. In Luckett, P. (ed.): *Reproductive Biology of the Primates*, *Contributions to Primatology*, *Vol. 3*, S. Karger, 1974, pp. 235-257.

Vaitukaitis, J. L. and Ross, G. T.: Hormonal control of ovulation. In *Gonadotropins in Female Infertility*, Amsterdam, Excerpta Medica, 1973, pp. 15-21.

NICHD Annual Report
Behavioral Biology Branch
July 1, 1973 through June 30, 1974

Section on Neurobiology

The professionals in this section include Drs. Phillip Nelson, Bruce Schrier, Earl Giller, Bruce Ransom, Ronald Fisk, Maryanna Henkart, Elaine Neale, Joseph Neale and Anthony Breuer. The guest workers are Drs. Clifford Christian and Wilfried Dimpfel.

1. Well differentiated neurons from the fetal rat brain have been grown in cell culture by a culture method involving the use of fluorodeoxyuridine and horse serum. We have begun to characterize and categorize these brain cells, on the basis of their morphology in the living state and after selective silver staining. Intracellular recordings revealed neuronal electrical excitability and generation of excitatory and inhibitory synaptic potentials as well as chemosensitivity to microiontophoretically applied neurotransmitters. Over 20% of the brain cells studied exhibited inhibitory postsynaptic potentials (IPSPs) and glycine and gamma-aminobutyric acid (GABA) both produced membrane changes consistent with their functioning as inhibitory transmitters. These cultures are being compared to cultures grown in the absence of fluorodeoxyuridine with regard to the biosynthesis of transmitter-related enzymes. Cells from the cerebral cortex and the remainder of the brain can be cultured independently and further regional fractionation of the brain is contemplated so that specific neuronal populations will become available for study.

2. Chemosensitivity and specific patterns of synaptic interactions have been explored in some detail in cultures of mouse spinal cord and dorsal root ganglion cells. Glutamic acid produces depolarizing, excitatory responses in a majority of spinal cord (SC) cells. The membrane change produced by glutamate is, however, different from that produced by the natural excitatory synaptic transmitter so that our data do not support the hypothesis that glutamate may serve as the excitatory transmitter. In complete growth medium hyperpolarizing inhibitory responses to GABA were found but no responses were seen to applied glycine in contrast to the situation with brain cells. IPSPs were present in less than 1% of cells studied in complete growth medium (which contains about 0.5 mM glycine). When cells are examined in glycine free medium responses to iontophoretically applied glycine can be demonstrated.

3. An interaction between spinal cord cells and muscle cells has been discovered in which the activity of the enzyme choline acetyltransferase (CAT) in nerve cells is augmented by greater than 10-fold when these cells are co-cultured with muscle cells. This enzyme is responsible for the synthesis of acetylcholine, the transmitter at nerve-muscle junctions, so the interaction is of functional significance. Medium in which muscle cells have been grown can partially produce the effect, so some diffusible substance is at least in part involved and experiments are in progress to define the nature of this inducing material. Enzymes other than CAT are either changed very little or substantially reduced, as in the case of glutamic acid decarboxylase, when nerve is co-cultured with muscle.

4. In collaboration with Dr. Klein we have found that rat pineal organs contain little if any activity of choline acetyltransferase. The apparent activity of that enzyme reported in pineals by another laboratory we found to be due to carnitine acetyltransferase. We have also found a very significant level of activity of glutamic acid decarboxylase in pineal organs. This enzyme, which forms the inhibitory neurotransmitter gamma-aminobutyric acid, had not been previously reported in pineal glands.

5. Adenosine stimulates the accumulation of intracellular cyclic adenosine monophosphate (cAMP) in brain and in glial tumor cells. Dr. Schrier, in conjunction with Dr. A. Gilman, has shown, using tritium labelled 2-chloroadenosine, that this response is receptor-mediated and is not due to some metabolic mechanism. The role of nucleotides in nerve transmission has received considerable experimental attention in the past few years and this adenosine response in glia may represent a mechanism related to the important problem of neuron-glia interaction.

6. Correlative electrophysiological and electron microscopic studies on neuroblastoma and hybrid cell lines with the Laboratory of Biochemical Genetics, NHLI, have centered on an extensive search for synapse formation by these cells. A number of clonal cell lines have been co-cultured with spinal cord, brain, cardiac and skeletal muscle as well as in various combinations of the clones. To date these studies have been negative, although the various neuroblastoma lines have been shown to exhibit essentially all known steps required for synapse formation. The problem is of sufficient importance to warrant continued efforts with some modified culture techniques.

7. A combined morphologic and electrophysiologic study has been made addressing what we felt are two inter-related problems. 1) What are mechanisms responsible for coupling between events initiated at the surface membrane of a cell and subsequent changes occurring in intracellular structure and metabolism. 2) What are important mechanisms involved in the development of cellular extension such as neurites or processes of other cells such as fibroblasts? L-cells develop processes under a number of well-defined conditions such as incubation with cAMP as has been described by others. The membrane properties of treated cells are changed from the normal; the transmembrane potential and membrane conductance are both increased. This sort of change can also be elicited by intracellular injection of Ca^{++} ions. Morphologically, an increase in subsurface cisternae are seen at the tips and at the base of processes. The processes contain both microtubules and microfilaments and the mitochondria and long tubular elements of smooth endoplasmic reticulum in the processes are oriented along the fibrous elements. A number of agents have been investigated with regard to their effects on membrane properties and ultrastructural features of the cells. The results are complex but we hope to develop a comprehensive model for the cellular activities involved in process formation and their regulation and to test the model in experiments with differentiating neurons in cell culture.

8. Light microscopical time lapse photography has been used to study certain aspects of transport of material within axons and other cellular processes. Particles can be seen to move in such processes and this motion has been

quantitated in spinal cord, brain and dorsal root ganglion cells, in various types of fibroblasts, including L-cells or HeLa cells and in neuroblastoma cells. The particles move at from 1-3 μ/sec and this rate is little changed over a temperature range of 29-38°C, or a pH range from 5.3 to 9.9. Both colchicine, which disrupts microtubules, and an agent that increases intracellular movement of calcium ions reduce or abolish particle movement.

9. A number of enzyme markers have been used in characterizing membrane preparations made from cell lines. Membrane preparations of L-cells, glial cells and neuroblastoma have been analyzed with disc gel electrophoresis. Glial cells have been chosen for intensive study since they give relatively good membrane preparations and because they have been shown to give a prompt metabolic response to adrenergic agents. Changes in membrane proteins that can be iodinated enzymatically occur within 2 hours and the mechanism of this prompt alteration is being investigated.

10. The fine structure of junctions between the surface membrane and subsurface cisternae of the endoplasmic reticulum in neurons has been found to resemble analogous structures in muscle cells. In the muscles studied these structures are the likely site of excitation-contraction coupling and the similarities noted strengthen the hypothesis that subsurface cisternae represent an important surface-to-cytoplasm coupling system in neurons.

Section on Functional Neurochemistry

The professionals in this section include Drs. Gainer, Barker, Sarne and Loh.

Extensive investigations of identifiable neurons in *Otala lactea* and *Aplysia californica* revealed highly specific patterns of protein synthesis correlated with specific cell functions. More elaborate studies, using gel systems with higher resolutions have allowed us to obtain better characterization of single cell protein synthesis profiles for future modulation studies. We have concentrated on a group of neurosecretory cells, R_{3-14}, in *Aplysia*, which appear to be involved in the feeding behavior of this animal, and the effects of this behavior on the electrical activity and synthesis patterns of these cells is currently under investigation. In previous studies we considered the effects of synaptic stimulation on the regulation of protein synthesis in an identified neurosecretory cell found in the mollusca, *Aplysia californica*. Comparisons of the protein synthesis patterns of synaptically inhibited neurons and spontaneously active control neurons showed that synaptic inhibition produced a selective 30% decrease in the synthesis of the 12,000 dalton peak. The putative inhibitory transmitter, dopamine, produced the same selective inhibition when added to the bathing medium. Depolarization of the cell by increasing the potassium ion concentration of the medium, selectively stimulated the synthesis of the 12,000 dalton protein by 50-70%. Thus, the rate of synthesis of the 12,000 dalton protein in R_{15} may be regulated by the membrane potential of the cell, which in turn is under control of a dopaminergic inhibitory synapse. Further studies on this cell have shown that the protein which is modulated by synaptic input has a high turnover rate, although changes in the decay processes cannot account for the results. Thus, it appears that specific synthesis is modified by this input. The rapid turnover of this protein has prompted us to initiate an electron microscopic autoradiographic study with Dr. Elaine Neale in order to identify the sub-cellular organelle which contains this protein.

Studies on the protein synthesis of the giant axon of the Squid (in collaboration with Dr. R. Lasek, at the Marine Biological Laboratory, Woods Hole, Massachusetts) have led to the observations that the axon's newly synthesized protein has its origin in the Schwann cell layer immediately surrounding the axon. We have considerable evidence for the intercellular transfer of these proteins (ranging from 0.5 million to < 12,000 daltons) and now are studying the regulation mechanisms underlying these glial-neuronal interactions and the biological significance of these transported proteins. Other studies on the squid axon (with Dr. Tasaki) have revealed a small, labile protein on the inner surface of the axon membrane which is affected by depolarization.

Our electrophysiological studies of the above-mentioned neurosecretory cells have revealed that divalent cations play a major and important regulatory role in both the bursting pacemaker potential activity of these cells and their seasonal modulation. As part of this project, we have concluded from our intracellular studies that bursting pacemaker potential activity is due predominantly to a time- and voltage-dependent potassium conductance, modulated by divalent cations and superimposed upon a constant sodium conductance. In collaboration with Dr. T. Smith, we have applied the voltage clamp technique to an investigation of the ionic currents involved at various phases of the bursting pacemaker potential cycle. Our results indicate that the current flow during the depolarizing phase of the cycle is predominantly inward, being predominantly dependent on both external sodium and divalent cation concentration. From these findings we have concluded that bursting pacemaker potential activity is primarily dependent on two membrane properties: (1) a voltage dependent inward divalent (calcium and magnesium) - monovalent cation (sodium) current closely coupled to (2) a voltage-dependent outward potassium current. These studies have demonstrated an important regulatory role for divalent cations both in the seasonal modulation of the neurosecretory cell's rhythm and the bursting pacemaker potential itself. These findings have led us to the study of hormonal effects on the electrophysiology and protein synthesis patterns of specific neurons. Thusfar, we have found that aldosterone as well as other corticosteroids inhibit bursting pacemaker activity in a specific snail neuron (cell 11), whereas vasopressin dramatically augments such activity in a highly specific manner. Furthermore, we have isolated an active peptide fraction from the brains of Aplysia and Otala which behaves similarly to that of vasopressin.

In collaboration with Drs. M. Brightman and Prescott, we have initiated a study correlating the morphological changes in neurosecretory neurons with their electrical, metabolic, and secretory activity. This study has shown the morphological consequences of calcium dependent exocytosis in neurosecretory endings, and we have also found a new neurosecretory cell in the sheath of the Aplysia ganglion.

These various findings have led us to conclude that the molluscan nervous system represents an excellent model for not only excitation-metabolic coupling studies, but also as a model of hypothalamic regulatory systems in mammals. The neuron upon which we are focusing our efforts, is modulated by dopaminergic synaptic input, and appears to receive long-tern negative feedback via circulatory steroids, and positive feedback by small peptides. Thus, in this single neuron, there resides a wide range of phenomenology characteristic of hypothalamic nuclei, which are amenable for detailed study in this model system. We believe that such model systems are essential for the analysis of regulatory mechanisms in neurons and the developing nervous systems.

Section on Brain and Behavior

The professionals in this section include Drs. Symmes, Newman and Alexander. Mrs. Riggs has contributed under a Professional Services Contract.

1) Single unit recording: During the last half of FY 74 a study was begun to investigate the neurophysiological and functional properties of a major pathway from the lateral frontal cortex into the temporal lobe. This pathway has been defined by anatomical methods, and the possibility that it may play a role in the processing of complex auditory input has been suggested by several behavioral and evoked potential studies.

The initial phase of this study involves testing the hypothesis that some auditory cortex neurons receive input both from the ear and from the frontal cortex. Single unit recordings in unanesthetized squirrel monkeys have been initiated using established techniques in this laboratory, and the influence on such cells of electrical stimulation of lateral frontal cortex is being analysed.

During this year considerable effort was devoted to developing computer programs that test statistically the complex patterns of cell discharges that result from the stimuli we employ. We now have available techniques for quantitatively comparing response patterns and will apply these to both current and previously obtained data.

Experiments were also undertaken to establish the neuropharmacological mechanisms underlying unit responsiveness to vocalizations. Iontophoretic application of putative neurotransmitters was paired with presentation of vocalizations. The major finding was that gamma-aminobutyric acid (GABA) depressed spontaneous unit activity to a greater extent than stimulus bound activity.

2) Behavioral Training: The emphasis in our continuing efforts to develop and exploit behavioral techniques has been on optimizing methods for food reward training as opposed to avoidance methods as used in prior studies. The objectives are 1) to replicate the finding that squirrel monkeys easily discriminate variant forms of the Isolation Peep call; 2) to conduct unit recording studies in monkeys which are performing various discrimination tasks.

The results obtained to date suggest that long training periods are required compared with avoidance methods, and efforts are underway to isolate the relevant training variables.

3) Analysis of Vocal Output: An extensive quantitative analysis of the variability in production of Isolation Peeps by captive squirrel monkeys has

been undertaken during FY 74. The existence of individual specificity in variant form, or at least of limited variability about a few "template" variants, has been hypothesized from visual inspection of sound spectrograms. Computer programs have been developed to permit large scale processing of these data, and approximately 2000 calls from 20 monkeys are being analysed. It is anticipated that this analysis will be completed early in FY 75.

Analysis of vocalizations emitted by paired female squirrel monkeys revealed different references in usage of Twitter variants and of the Traller call type. Some of these differences were ascribable to the dominance position of the vocalizer. A form of vocal "duetting" was discovered, involving the Err Chuck call, in which one animal produces the Err, the other producing the Chuck.

Collaborative studies have been initiated on the possible genetic basis of structural differences in Isolation Peep variants. These studies will continue, focussing on analysis of Isolation Peep structure from squirrel monkeys originating in different regions of their natural range, and hybrids produced by pairing individuals from geographically separate populations.

Serial No. HD-BB4
1. Behavioral Biology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Information processing in the central auditory system of mammals.

Previous Serial Number: Same

Principal Investigators: John D. Newman, Ph.D.
David Symmes, Ph.D.

Other Investigators: Garrett Alexander, M.D.
Garland Riggs

Cooperating Units: Dr. T. C. Jones, New England Regional Primate Research Center, Southborough, Mass.
Dr. Steven Foote, Laboratory of Neuropharmacology, National Institute of Mental Health, St. Elizabeth's Hospital, Washington, D.C.

Man Years:

Total:	3.7
Professional:	3.0
Others:	0.7

Project Description:

Objectives: This project is concerned with the processing of complex sounds by the primate cerebral cortex and related brain structures. Special attention is being paid to sounds used by primates in intra-specific communication, to determine which cues are used by the brain and the animal to distinguish between the various elements of the communication repertoire.

Methods Employed and Major Findings: 1) Single unit recording: During the last half of FY 1974 a study was begun to investigate the neurophysiological and functional properties of a major pathway from the lateral frontal cortex into the temporal lobe. This pathway has been defined by anatomical methods, and the possibility that it may play a role in the processing of complex auditory input has been suggested by several behavioral and evoked potential studies.

The initial phase of this study involves testing the hypothesis that some auditory cortex neurons receive input both from the ear and from the frontal cortex. Single unit recordings in unanesthetized squirrel monkeys have been initiated using established techniques in this laboratory, and the influence on such cells of electrical stimulation of lateral frontal cortex is being analysed.

During this year considerable effort was devoted to developing computer programs that test statistically the complex patterns of cell discharges that result from the stimuli we employ. We now have available techniques for quantitatively comparing response patterns and will apply these to both current and previously obtained data.

Experiments were also undertaken to establish the neuropharmacological mechanisms underlying unit responsiveness to vocalizations. Iontophoretic application of putative neurotransmitters was paired with presentation of vocalizations. The major finding was that gamma-aminobutyric acid (GABA) depressed spontaneous unit activity to a greater extent than stimulus bound activity.

2) Behavioral Training: The emphasis in our continuing efforts to develop and exploit behavioral techniques has been on optimizing methods for food reward training as opposed to avoidance methods as used in prior studies. The objectives are 1) to replicate the finding that squirrel monkeys easily discriminate variant forms of the Isolation Peep call; 2) to conduct unit recording studies in monkeys which are performing various discrimination tasks.

The results obtained to date suggest that long training periods are required compared with avoidance methods, and efforts are underway to isolate the relevant training variables.

3) Analysis of Vocal Output: An extensive quantitative analysis of the variability in production of Isolation Peeps by captive squirrel monkeys has been undertaken during FY 1974. The existence of individual specificity in variant form, or at least of limited variability about a few "template" variants, has been hypothesized from visual inspection of sound spectrograms. Computer programs have been developed to permit large scale processing of these data, and approximately 2000 calls from 20 monkeys are being analyzed. It is anticipated that this analysis will be completed early in FY 1975.

Analysis of vocalizations emitted by paired female squirrel monkeys revealed different references in usage of Twitter variants and of the Traller call type. Some of these differences were ascribable to the dominance position of the vocalizer. A form of vocal "duetting" was discovered, involving the Err Chuck call, in which one animal produces the Err, the other producing the Chuck.

Collaborative studies have been initiated on the possible genetic basis of structural differences in Isolation Peep variants. These studies will continue, focussing on analysis of Isolation Peep structure from squirrel monkeys originating in different regions of their natural range, and hybrids produced by pairing individuals from geographically separate populations.

Significance to Biomedical Research and the Program of the Institute: Our demonstration of the role of call type variants in vocal communication and their perception by the auditory cortex has provided a new perspective to non-human primate communication. Based on these findings, there appears now a greater possibility of relating cerebral processing of communication sounds in nonhuman primates to basic mechanisms of speech perception in humans.

Proposed Course: The work to date in this project has produced a substantial description of the encoding of vocalizations by neurons in the primate auditory cortex, and suggested some behavioral correlates of this neural processing. Future work may now begin to deal with other related questions: 1) the role of early experience and inheritance in the establishment of neuronal specificity to vocalizations; 2) the functional relationships of other brain systems (especially the frontal-limbic system) to the auditory cortex; 3) the relationship between chemical specificity (as defined by differential sensitivity to various transmitter substances) and sensory specificity (as defined by differential specificity to vocalizations). The ontogeny of vocal communication in primates is an area of considerable promise. We expect to combine behavioral studies of early vocal communciation with studies of the development of neurophysiological mechanisms relevant to communication.

Honors and Awards: None

Publications:

1. Newman, J. D. and Symmes, D.: Vocal pathology in socially deprived monkeys. Develop. Psychobiol., in press.
2. Newman, J. D. and Wollberg, Z.: Responses of single neurons in the auditory cortex of squirrel monkeys to variants of a single call type. Exptl. Neurol. 40: 821-824, 1973.
3. Symmes, D. and Newman, J. D.: Discrimination of isolation peep variants by squirrel monkeys. Exptl. Brain Res. 19: 365-376, 1974.

Serial No. HD-BB5
1. Behavioral Biology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Neurobiologic studies of neuronal and other cell types in cell culture.

Previous Serial Number: Same

Principal Investigators: Phillip G. Nelson, M.D., Ph.D.
Bruce K. Schrier, M.D., Ph.D.

Other Investigators: Earl L. Giller, M.D., Ph.D.
Anthony C. Breuer, M.D.
Clifford N. Christian, Ph.D.
Joseph H. Neale, Ph.D.
Elaine A. Neale, Ph.D.
Bruce R. Ransom, M.D., Ph.D.
Harris R. Fisk, M.D., Ph.D.
Maryanna Henkart, Ph.D.
Wilfried Dimpfel, D.V.M.
Earl W. Godfrey

Cooperating Units: A. G. Gilman, Department of Pharmacology, Univ. of Virginia, School of Medicine, Charlottesville, Virginia.
J. Minna, X. Breakefield and M. Nirenberg, Laboratory of Biochemical Genetics, National Heart and Lung Institute

Man Years:

Total:	13.5
Professional:	9.4
Others:	4.1

Project Description:

Objectives: To analyze the differentiation of neurobiologically important cell properties in a number of cell culture systems. We have continued our work in developing preparations which will represent as completely as possible normal nervous system constituents. During the past year a more analytical approach to specific neurobiologic problems has been possible and we have addressed the following questions: 1) What trophic interactions between muscle and spinal cord cells occur in culture and can similar trophic effects be demonstrated between different regions of the nervous system. 2) Will continuous lines of neurons establish synaptic or other relations with potential target cells. 3) What are the membrane mechanisms responsible for the chemosensitivity of nerve cells to various putative neurotransmitters and how closely do these mechanisms correspond to those involved in synaptic transmission. 4) What are the mechanisms involved in growth of cellular processes and in transport of material within the processes.

Methods Employed: Most of the culturing, biochemical, electrophysiologic and morphologic techniques have been detailed in previous reports. In addition, some specific methodologic advances have been made: 1) Cell culture techniques which allowed differentiation of neurons on glass coverslips, which allowed fluorescence, Nomarski and other high resolution light microscopy. 2) Techniques for time-lapse photomicrography at high magnification with Nomarski differential interference contrast microscopy, and for analysis on these films of particle motion within cell processes using computer programs developed for that purpose. 3) Methods for preparation and purification of plasma membranes from neuroblastoma and glioma cells.

Major Findings: a) Clones of hybrid cells formed from neuroblastoma and L-cell parent lines have been found to have electrically active membranes, suggesting a positive rather than negative control over expression of this genetic potential. Spike electrogenesis was expressed in conjunction with membrane sensitivity to acetylcholine and the presence of the enzyme acetylcholinesterase. Electrically active membranes and acetylcholine sensitivity have also been found in hybrid cells formed from neuroblastoma and Chinese hamster central nervous system. To date, no evidence of synapse formation between cells in the hybrid clones or in combined cultures of neuroblastoma or hybrids with effector cells (skeletal, smooth or cardiac muscle) has been obtained.

An evaluation of the purity of membrane preparations from C-6 glioma cells by several different enzymatic tests has shown that a purification of 4- to 6-fold for membrane enzymes has been achieved. With this degree of purification and with an ^{125}I-labelling marker for external plasma membrane proteins, an examination of the dynamics of membrane components has been initiated.

b) By use of tritium-labelled 2-chloroadenosine it was shown that the adenosine-mediated stimulation of cyclic AMP accumulation in brain cells and many established cell lines in culture occurs via a receptor-mediated rather than a metabolic mechanism. This demonstration answered a controversy which had been extant since discovery of the adenosine response in brain slices a decade ago.

c) Additional data have been obtained on the electrophysiologic, morphologic and biochemical characteristics of fetal rat brain cells in surface culture. Application of a culture regimen involving treatment of rat brain cell cultures with the DNA synthesis inhibitor fluorodeoxyuridine and subsequent maintenance of the cells in medium with 10% horse serum, resulted in the appearance of highly differentiated populations of nerve cells in this system. The neurons in such cultures were large, electrically active and stained selectively with silver. Complex patterns of action potentials as well as inhibitory and excitatory post-synaptic potentials and sensitivity to several iontophoretically applied putative neurotransmitters were found. Using microscopic techniques such as phase contrast, silver staining, Nomarski optics and electron microscopy, the morphology of individual neurons, as well as synaptic relationship between cells are being examined. Further studies of the effects of inhibition of DNA synthesis on the development of neurotransmitter-related enzymes may clarify whether neurons are multiplying in culture.

d) Movement of particles in cell processes has been studied in a number of neuronal and non-neuronal cell types. These have demonstrated that in these various cell types the particles: 1) translocate at average velocities from 1 to 3 μm/sec., 2) may move forward, stop and reverse their course, 3) demonstrate little velocity change over a range of temperatures from 29°C to 38°C and a pH range of 5.3 to 9.0 and 4) become more regular in their motion as the cells mature in culture. A number of pharmacologic agents have been examined for their effects on particle movement, including colchicine and the artifical calcium ionophore A23187, both of which impair such translocation.

e) We have continued to evaluate the development and function of spinal cord cells (SC) in culture, and have found some marked trophic effects of muscle cells (M) on SC. Co-culture of SC on established cultures of M resulted in a 10- to 17-fold increase in the activity, relative to SC alone, of the enzyme choline acetyltransferase (CAT). CAT is responsible for the synthesis of acetylcholine, the presumed transmitter at neuromuscular junctions. This phenomenon was shown not to be due to increased plating efficiency of SC on M, was temporally associated with the formation of functional neuromuscular junctions in the cultures, and occurred concurrently with a 4-fold reduction in activity of the enzyme glutamic acid decarboxylase (GAD). This enzyme is responsible for synthesis of GABA, a putative inhibitory neurotransmitter in spinal cord. Other spinal cord and muscle marker enzymes changed little, if any, in combined SC-M cultures, indicating some specificity of this trophic effect. These trophic effects on both enzymes could be reproduced by inoculation of M onto SC as well as the reverse, and the magnitude of the effect was dependent on the "dose" of muscle. Conditioned medium from muscle cell cultures had a similar, dose-dependent stimulatory effect on CAT activity, thus providing the opportunity to purify and characterize the factor(s) responsible for this effect. The negative trophic effect on GAD activity, which may have been related to loss of some medium-sized neurons in combined SC-M cultures, was not reproduced by conditioned medium or by inoculating SC onto heart muscle cells in culture. This suggested that some specific cell contact phenomena might be involved.

Work with electrophysiologic characteristics of SC cultures has involved synaptic interactions which occur between specific pairs of neurons. Reciprocal excitatory connections have been observed between SC cells. Dorsal root ganglion (DRG) neurons can form excitatory contacts with SC, but DRG cells do not appear to receive synaptic connections from other cells. DRG cells were uniformly insensitive to iontophoretically applied glutamate and aspartate, while both amino acids were excitatory for some SC neurons. In cells responsive to glutamate, localized areas of increased sensitivity were found along neurites. The reversal potentials at some DRG-SC synapses were found to be different from those for glutamate, suggesting that amino acid is not the transmitter at those junctions. Detailed studies with iontophoretically applied glycine and GABA showed that DRG neurons were unresponsive. A high percentage of SC neurons responded to GABA with a large increase in membrane conductance. No glycine responses and only rare IPSP's were seen in cultures in normal medium (with 0.4 mM glycine). When mature cultures were switched acutely to a defined salt solution containing no amino acids, glycine responses were commonly observed and had the same equilibrium potential as the GABA responses, but IPSP's were not observed acutely. Preliminary data suggests that IPSP's can be detected when cultures are grown in glycine free medium. Experiments are underway to explore the implications of this result as applied to the formation and/or maintenance of specific synaptic contacts.

Significance to Biomedical Research and the Program of the Institute: The regulatory role of the nervous system is of great importance in the process of normal tissue development. Metabolic interactions between nerve cells and target cells are well documented in a number of intact systems but the analysis of the mechanisms responsible is difficult. The analytic potential of the culture systems is considerable and we now have evidence for powerful intercellular interactions occurring in these systems. Explication of the mechanisms involved in these interactions would be of substantial value. Our increasing knowledge of the normal neurobiology of several cell culture systems represents a necessary prerequisite for the study of the important determinants of some selected neuropathologic conditions.

Proposed Course: Most of the studies listed above are continuing; specifically, these include: 1) evaluation of the electrophysiologic, morphologic and biochemical properties and genetic control in established cell lines from the nervous system. This will include a continued effort to find conditions under which such cells will form functional synapses. 2) Continued investigation of the fetal brain cell culture system; including examination of uptake systems for neurotransmitter molecules, the specificity of synapse formation and biochemical evidences of interaction between various brain areas in culture and efforts to correlate morphologic, biochemical and electrophysiologic characteristics of specific cells and cell types in the cultures. 3) Continued efforts to determine the effects of the external milieu on the membrane responses of spinal cord and DRG neurons. 4) Investigations into the mechanism(s) by which skeletal muscle cells and medium conditioned by them exert trophic effects upon spinal cord cells in culture. In addition, we propose to begin studies of normal vs. abnormal developmenta and function using mutant mice. The following specific projects are under consideration: a) Comparisons of the biochemistry and electrophysiology of cortex cell and brain cell cultures from normal and genetically epileptic mice. A variety of mutants are available; the most profitable of these to use may be "Lethargic" and "Tottering". b) Comparison of the trophic effects of muscle cells on spinal cord cells in cultures from normal as well as dystrophic mice. The mutant to be used would be "Dystrophy" - an apparent analog of human Duchenne muscular dystrophy. c) A dissection of the metabolic events involved in myelin formation in brain cell cultures derived from animals deficient in myelin formation. There are three mouse mutants in which impaired myelin formation occurs, and these three appear to involve different genetic loci. In this instance, use of the abnormal system is suggested as a probe for understanding normal development.

Honors and Awards: None

Publications:

1. Nelson, P. G.: Electrophysiologic studies of normal and neoplastic cells in tissue culture. In Sato, G. (ed.): Tissue Culture of the Nervous System. New York, Plenum Publishing Corp., 1973, pp. 135-160.

2. McMorris, F. A., Nelson, P. G. and Ruddle, F.: Contributions of Clonal Systems to Neurobiology. A Report Based on an NRP Work Session, Volume 11, 1973, pp. 414-536.

3. Giller, E. L., Schrier, B. K., Shainberg, A., Fisk, H. R. and Nelson, P. G.: Choline acetyltransferase activity is increased in combined cultures of spinal cord and muscle cells from mice. Science 182: 588-589, 1973.

4. Schrier, B. K. and Shapiro, D. L.: Effects of N^6-monobutyryl-cyclic AMP on glutamate decarboxylase activity in fetal rat brain cells and glial tumor cells in culture. Exptl. Cell Res. 80: 459-462, 1973.

5. Schrier, B. K. and Wilson, S. H.: Investigation of methods for measurement of radioactivity in tritiated DNA and applications to assays for DNA polymerase activity. Analyt. Biochem. 56: 196-207, 1973.

6. Spector, I., Kimhi, Y. and Nelson, P. G.: Tetrodotoxin and cobalt blockade of neuroblastoma action potentials. Nature New Biol. 246: 124-126, 1973.

7. Schrier, B. K. and Thompson, E. J.: On the role of glial cells on the mammalian nervous system: uptake, excretion and metabolism of putative neurotransmitters by cultured glial tumor cells. J. Biol. Chem. 249: 1769-1790, 1974.

8. Fischbach, G. D. and Dichter, M. A.: Electrophysiologic and morphologic properties of neurons in dissociated spinal cord cell cultures. Devel. Biol. 37: 100-116, 1974.

Serial No. HD-BB8
1. Behavioral Biology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Correlative neurochemical and electrophysiological analyses of individual, identifiable neurons in molluscan nervous systems.

Previous Serial Number: Same

Principal Investigator: Harold Gainer, Ph.D.

Other Investigators:
Jeffery L. Barker, M.D.
Yoke P. Loh, Ph.D.
Yosef Sarne, Ph.D.

Cooperating Units:
Thomas Smith, M.D., Laboratory of Neurophysiology, NINDS
Ichiji Tasaki, M.D., Laboratory of Neurobiology, NIMH and Marine Biological Laboratory, Woods Hole, Mass.
Elaine Neale, Ph.D., Behavioral Biology Branch, NICHD
Milton Brightman, Ph.D. and Lisa Prescott, M.D., Laboratory of Neuropathology, NINDS
Raymond Lasek, Ph.D., Case-Western Reserve Univ., Department of Anatomy, School of Medicine and Marine Biological Laboratory, Woods Hole, Mass.
Eugene Straus, M.D., Mt. Sinai School of Medicine, Bronx V. A. Hospital, New York, New York.

Man Years:

Total:	3.9
Professional:	3.1
Others:	0.8

Project Description:

Objectives: To study the regulatory mechanisms underlying specific protein synthesis and electrophysiological properties of identifiable neurons.

Methods Employed: Several methods for the separation and analysis of proteins synthesized by single neurons have been developed in this laboratory. These include dissection of the individual cells from the ganglion under a low-power microscope with the use of fine needles as well as mechanical homogenization of the cells and chemical solubilization of their proteins. The analytical tools include (1) general electrophoresis of proteins in which their separation is based on molecular weight and charge, (2) polyacrylamide gel electrophoresis of proteins in sodium dodecyl sulfate (separation based on molecular weight only) and (3) isoelectric focusing of proteins (separation based on charge only). All of these separation techniques have been developed and used on both the macro and the micro scale, and together with radioisotopes of amino-acids and other labeled precursors, considerable information has been gathered about the protein metabolism of single neurons. Other "single-cell" chemical methodologies being developed in our laboratory include (1) ultracentrifugation of membranes and cytoplasmic contents, (2) methods in lipid and liproprotein biochemistry, (3) nucleotide biochemical techniques, (4) intracellular isotope injection techniques for axoplasmic transport studies, and (5) column chromatography specifically adapted to the isolation of biologically active oligopeptides.

Two experimental preparations are being utilized for these studies. The isolated brain of the land snail, *Otala lactea*, and the abdominal ganglion of the sea hare, *Aplysia californica*. Both nervous systems contain large, identifiable neurons which are appropriate for combined electrophysiological-neurochemical studies. Conventional electrophysiological recording and stimulating methods are also employed in these studies. Some of these methods are (1) intracellular recording from individual, identified cells in molluscan ganglia, (2) stimulation techniques for the purpose of studying synaptic transmission, (3) manipulation of the ionic environment for the purpose of studying membrane potentials and their ionic bases and (4) voltage-clamp techniques for the purpose of studying the ionic currents involved in membrane potential changes.

In addition, the giant axon of the squid has been intracellularly perfused with protein radiodination solutions (i.e., lactoperoxidase techniques) in a search for functionally significant nerve membrane proteins, as well as in a study of Schwann-cell to axon interactions.

Major Findings: Extensive investigations of identifiable neurons in *Otala lactea* and *Aplysia californica* revealed highly specific patterns of protein synthesis correlated with specific cell functions (see Gainer and Wollberg, 1974). More elaborate studies, using gel systems with higher resolutions (work by Dr. Loh), have allowed us to obtain better characterization of single cell protein synthesis profiles for future modulation studies. In the latter case, we have concentrated on a group of neurosecretory cells, R_{3-14}, in *Aplysia*, which appear to be involved in the feeding behavior of this animal, and the effects of this behavior on the electrical activity and synthesis patterns of these cells is currently under investigation. In previous studies we considered the effects of synaptic stimulation on the regulation of protein synthesis in an identified neurosecretory cell found in the mollusca, *Aplysia californica* (Gainer and Barker, in preparation). The bursting pacemaker neuron, R_{15}, in the abdominal ganglion of *Aplysia californica* exhibits a specific pattern of protein synthesis when incubated in ^{3}H-Leucine. SDS-polyacrylamide gel electrophoresis shows that 20-25% of the *de novo* synthesized proteins of the cell is accounted for by a single molecular weight class (around 12,000 daltons). Stimulation of the branchial nerve for 5 hours at a rate of 5/sec, hyperpolarized the cell and blocked all spontaneous spike activity. Comparisons of the protein synthesis patterns of synaptically inhibited neurons and spontaneously active control neurons showed that synaptic inhibition produced a selective 30% decrease in the synthesis of the 12,000 dalton peak. The putative inhibitory transmitter, dopamine, produced the same selective inhibition when added to the bathing medium. Depolarization of the cell by increasing the potassium ion concentration of the medium, selectively stimulated the synthesis of the 12,000 dalton protein by 50-70%. Thus, the rate of synthesis of the 12,000 dalton protein in R_{15} may be regulated by the membrane potential of the cell, which in turn is under control of a dopaminergic inhibitory synapse. Further studies on this cell have shown that the protein which is modulated by synaptic input has a high turnover rate, although changes in the decay processes cannot account for the results. Thus, it appears that specific synthesis is modified by this input. It may be that in such relatively short-term experiments changes will be observed only in proteins with high turnover rates, whereas longer-term experiments will be necessary to demonstrate alterations in more stable macromolecules. The rapid turnover of this protein has prompted us to initiate an electron microscopic autoradiographic study with Dr. Elaine Neale in order to identify the sub-cellular organelle which contains this protein. Furthermore, Dr. Sarne is attempting to isotopically label the proteins of a single neuron (R_{15} in Aplysia, cell 11 in Otala) in order to follow which proteins and subcellular organelles are transported into the axon, and are then subsequently secreted upon excitation of the neuron.

Studies on the protein synthesis of the giant axon of the Squid (in collaboration with Dr. R. Lasek, at the Marine Biological Laboratory, Woods Hole, Massachusetts) have led to the observations that the axon's newly synthesized protein has its origin in the Schwann cell layer immediately surrounding the axon. We have considerable evidence for the intercellular transfer of these proteins (ranging from 0.5 million to < 12,000 daltons, in preparation) and now are studying the regulation mechanisms underlying these glial-neuronal interactions and the biological significance of these transported proteins (see Lasek, et al, 1974). Other studies on the squid axon (with Dr. Tasaki) have revealed a small, labile protein on the inner surface of the axon membrane which is affected by depolarization (see Gainer, et al, Comp. Biochem. Physiol., 1974).

Our electrophysiological studies of the above-mentioned neurosecretory cells have revealed that divalent cations play a major and important regulatory role in both the bursting pacemaker potential activity of these cells and their seasonal modulation. As part of this project, we have concluded from our intracellular studies that bursting pacemaker potential activity is due predominantly to a time- and voltage-dependent potassium conductance, modulated by divalent cations and superimposed upon a constant sodium conductance. In collaboration with Dr. T. Smith, we have applied the voltage clamp technique to an investigation of the ionic currents involved at various phases of the bursting pacemaker potential cycle. Our results indicate that the current flow during the depolarizing phase of the cycle is predominantly inward, being predominantly dependent on both external sodium and divalent cation concentration. From these findings we have concluded that bursting pacemaker potential activity is primarily dependent on two membrane properties: (1) a voltage dependent inward divalent (calcium and magnesium) - monovalent cation (sodium) current closely coupled to (2) a voltage-dependent outward potassium current. These studies have demonstrated an important regulatory role for divalent cations both in the seasonal modulation of the neurosecretory cell's rhythm and the bursting pacemaker potential itself (see Barker and Gainer, 1973a, 1974a). These findings have led us to the study of hormonal effects on the electrophysiology and protein synthesis patterns of specific neurons. Thusfar, we have found that aldosterone as well as other corticosteroids inhibit bursting pacemaker activity in a specific snail neuron (cell 11), whereas vasopressin dramatically augments such activity in a highly specific manner (see Barker and Gainer, 1974b). Furthermore, we have isolated an active peptide fraction from the brains of Aplysia and Otala which behaves similarly to that of vasopressin (this work has been done by M. Ifshin).

In collaboration with Drs. M. Brightman and Prescott (freeze fracture and etching), we have initiated a study correlating the morphological changes in neurosecretory neurons with their electrical, metabolic, and secretory activity. This study has achieved one of its aims which was to show the morphological consequences of exocytosis (calcium dependent) in neurosecretory endings, and as a by-product we have discovered a new neurosecretory cell in the sheath of the Aplysia ganglion.

Significance to Biomedical Research and the Program of the Institute: The approach described above is specifically designed to correlate relevant biochemical changes taking place in the nervous system with its specific functional activity. The heterogeneity of nervous tissue makes the most direct approach to this problem a desirable one. In this approach individual identifiable neurons can be studied electrically and biochemically thereby providing a direct correlation between these events - and it is hoped ultimately an insight into what membrane phenomena in neurons lead to long-term intraneuronal biochemical changes. The molluscan nervous system represents an excellent model for not only excitation-metabolic coupling studies, but also as a model of hypothalamic regulatory systems in mammals. The neuron upon which we are focusing our efforts, is modulated by dopaminergic synaptic input, and appears to receive long-term negative feedback via circulatory steroids, and positive feedback by small peptides. Thus, in this single neuron, there resides a wide range of phenomenology characteristic of hypothalamic nuclei, which are amenable for detailed study in this model system. We believe that such model systems are essential for the analysis of regulatory mechanisms in neurons and developing nervous systems.

Proposed Course: In view of our objectives, we are currently moving in several directions: 1) To try long-term organ culture techniques on the snail brain so that we will then be able to study the long-term regulation of protein synthesis in specific neurons in this ganglion (e.g., the induction of cell 11's specific protein from aestivated animals). 2) The behavioral and electrophysiological regulation of R_{3-13}'s (Aplysia) specific neurosecretory proteins will be studied as mentioned above. 3) To evaluate which newly-synthesized proteins are secreted from specific neurons, and to determine in which subcellular organelles these proteins reside. This information will give further insight into the functions of these neurons. 4) In collaboration with Dr. E. Straus (Mt. Sinai Medical School) we are doing radioimmunoassays on the blood, ganglia, and single neurons of *Aplysia* and *Otala* in order to determine if the hormones released are comparable to human hormones. Thusfar, we have detected insulin and gastrin (but not growth hormone) at fasting human levels. 5) To expand our peptide analysis technology so as to be able to isolate biologically active neuropeptides and to determine their significance for neuronal activity and behavior. 6) To continue the analysis of cell-to-cell transfer of macromolecules in the Squid axon system. We plan to extend our studies to axoplasmic flow from the squid eye to nerve terminals in the optic lobe, and to determine whether specific proteins are released from synaptosomes upon electrical stimulation. 7) To extend our studies to a developing nervous system of a vertebrate (possibly the Mauthner cell found in the brains of fishes and amphibians).

Honors and Awards: None

Publications:

1. Barker, J. L. and Gainer, H.: Role of calcium in the seasonal modulation of pacemaker activity in a molluscan neurosecretory cell. *Nature* 245: 462-464, 1973a.

2. Barker, J. L. and Gainer, H.: Pentobarbital: Selective depression of excitatory postsynaptic potentials. *Science* 182: 720-722, 1973b.

3. Nicholl, R. A. and Barker, J. L.: Effect of strychnine on dorsal root potentials and amino acid responses in frog spinal cord. *Nature New Biology* 246: 224-225, 1973.

4. Barker, J. L. and Gainer, H.: Bursting pacemaker potential activity in a normally silent neuron. *Brain Res.* 65: 516-520, 1974a.

5. Gainer, H., Carbone, E., Singer, I., Sisco, K. and Tasaki, I.: Depolarization-induced change in the enzymatic radioiodination of a protein on the internal surface of the squid giant axon membrane. *Comp. Biochem. Physiol.* 47A: 477-484, 1974.

6. Barker, J. L. and Levitan, H.: Phenols: effects on membrane permeability of molluscan neurons. *Brain Res.* 67: 555-561, 1974.

7. Barker, J. L. and Levitan, H.: Studies on mechanisms underlying non-narcotic analgesia. *Adv. in Neurology* 4: 503-511, 1974.

8. Lasek, R. J., Gainer, H. and Pryzbylski, R.: Transfer of newly synthesized proteins from Schwann cells to the Squid giant axon. *Proc. Nat. Acad. Sci. U.S.*, in press, 1974.

9. Colburn, T. R., Wollberg, Z. and Gainer, H.: A logarithmic display of interspike-interval for long-term, patterned neural activity. *Medical and Biological Engineering*, in press, 1974.

10. Gainer, H. and Wollberg, Z.: Specific protein metabolism in identifiable neurons of *Aplysia californica*. *J. Neurobiol.*, in press, 1974.

11. Barker, J. L. and Gainer, H. Peptide regulation of bursting pacemaker activity in a molluscan neurosecretory cell. *Science*, in press, 1974.

Serial No. HD-BB9
1. Behavioral Biology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: A correlated study of the fine structure and the function of the endoplasmic reticulum (ER) of neurons.

Previous Serial Number: Same

Principal Investigator: Maryanna Henkart, Ph.D.

Other Investigators: Anthony C. Breuer, M.D.

Cooperating Units: Drs. T. S. Reese and D. M. D. Landis, Section on Functional Neuroanatomy, Laboratory of Neuropathology and Neuroanatomy, NINDS

Man Years:

Total:	0.8
Professional:	0.5
Others:	0.3

Project Description:

Objectives: To further develop and apply methods for demonstration with the electron microscope of sites of Ca binding or accumulation in order to explore the possibility that certain physiological and/or metabolic functions in neurons are regulated in coordination with membrane activity via Ca entry or release of Ca from internal stores.

Methods Employed: In general, the methods are the same as in the previous year. Emphasis has been placed on further substantiating the role of the ER in controlling the intracellular Ca concentration by extending the experiments with changes in ER morphology under various physiological conditions in both squid axon and *Aplysia* neurons. The identification of Ca-accumulation sites is being carried out with techniques involving fixation in the presence of various concentrations of divalent ions (Ca, Mg), substitution of more electron-opaque divalent ions (Sr, Co, La), and fixation in buffer solutions that should precipitate divalent ions of interest (PO_4). Dense deposits are identified as containing Ca by soaking thin sections of embedded material in solutions of Ca chelators and observing the disappearance of opaque deposits. The tissues are fixed under various physiological conditions produced by alteration of the ionic compositions of the physiological saline bathing the living material. The changes of morphology of the ER under these various conditions are analysed.

In collaboration with Drs. T. S. Reese and D. Landis freeze fracture studies have been done comparing the structure of junctions between surface membrane and sub-surface cisterns of ER in neurons with the structure of junctions between sub-surface cisterns and surface membranes in muscle where such junctions are thought to be the site of excitation-contraction coupling.

Major Findings: Alterations in the ionic composition of the artificial sea water which have been shown by others to increase the cytoplasmic Ca concentration and to increase the amount of Ca entering the cytoplasm in association with an action potential in squid axon also produce swelling of the ER and increase the opacity of the ER membranes. This supports the idea that Ca uptake may occur in the ER. Three further observations suggest that the ER may release Ca under the influence of changes in the membrane potential. 1) Others have shown that storage of axons in physiological solution in the cold increases the amount of Ca appearing in the cytoplasm with each action potential. We find that similarly stored axons have greatly swollen ER. 2) Cobalt which was shown by others to block the influx of Ca usually observed in association with depolarization produces swelling of the ER and increased opacity of the ER membrane. 3) In axons which have been treated with high Ca and have swollen ER, the ER is much reduced in volume after a brief depolarization in 90 mM K^+ artificial sea water or after a short period of repetitive stimulation.

A new intracellular compartment in Aplysia neurons has been identified as containing Ca. Pigment granules that are developmentally related to the endoplasmic reticulum contain a dense precipitate that is soluble in a Ca chelating solution. These pigment granules undergo a morphological change upon illumination. Others have shown that upon illumination the membrane potential and conductance to potassium ions increase in this neuron. Those membrane changes can be mimicked by the intracellular injection of Ca. These findings suggest that Ca may be released from the pigment granules by illumination.

Freeze-fracture studies show that the size and distribution of intramembranous particles in junctions between sub-surface cisterns of the endoplasmic reticulum and surface membrane are similar in muscle and neurons. This strengthens the analogy between the endoplasmic reticulum of neurons and the sarcoplasmic reticulum of muscle.

Significance to Biomedical Research and the Program of the Institute: The observations so far substantiate the analogy between the endoplasmic reticulum of neurons and the sarcoplasmic reticulum of muscle which is known to function as a Ca-sequestering and releasing system. It suggests that in nerve cells the ER may also be involved in coupling the electrical activity of the surface membrane to intracellular activities, the coupling being mediated by Ca released from the ER. If this is the case it could represent a critical control mechanism for a number of neuronal activities. Physiological studies in a number of laboratories have demonstrated that intracellular Ca can change the permeability properties of the cell membrane and, therefore, control the cell excitability. Our experiments and those of others suggest that intracellular Ca may be involved in phototransduction and light adaptation. Preliminary studies in this Branch (see particle transport project report) suggest that alterations in intracellular Ca concentration may affect the translocation of particles in neurites. Our experiments in another system (see L cell morphogenesis project report) suggest that the control of intracellular Ca concentration may be a key factor in regulation of changes in cell shape associated with differentiation in process-bearing cells. All of these cellular functions play important roles in the normal development and maintenance of neurons.

Proposed Course: A comparison of the effects of changes in intracellular Ca concentration and distribution on the fine structure of other neurons is planned. The intracellular calcium concentration has been reported to be a function of previous illumination and to control the level of light adaptation in the Limulus ventral photoreceptor. In collaboration with Dr. J. E. Brown we intend to see whether the endoplasmic reticulum of this neuron responds to changes in cytoplasmic Ca concentration in the same ways as the ER of the squid axon, and whether the ER may be the intracellular store of Ca^{++} suggested by his physiological experiments. It is desirable to identify directly the ionic composition of dense deposits produced by the various fixation techniques used in these studies. For this purpose we intend to use X-ray analysis which requires equipment not available in our laboratory. Discussions are now in progress with the owners and operators of several instruments that may be available for short term projects. Further study of the role of divalent ions in the movement of intra-axonal particles is also planned.

Honors and Awards: None

Publications: None

Serial No. HD-BB-10
1. Behavioral Biology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Changes in membrane properties and fine structure associated with cell morphology.

Previous Serial Number: New Project.

Principal Investigators: Maryanna Henkart, Ph.D.
Phillip G. Nelson, M.D., Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.3
Professional:	1.0
Others:	0.3

Objectives: Various cultured cell lines have been shown to stop dividing and "differentiate", that is, to undergo some morphological change, as a result of various alterations in the culture medium. X-irradiated L-cells, a cell line of mouse fibroblast origin, change in 3-6 hours from a round or flattened polygonal form to a shape characterized by various arrangements of elongate processes upon treatment with 1 mM dibutyryl cyclic AMP (db cAMP). The objective of this project is to elucidate the mechanisms underlying this cellular remodeling which resembles cellular differentiation. A second and related goal is to use db cAMP-induced process formation in L-cells as a model for process formation in neurons and/or glia.

Methods Employed: L-cells of strain LM (TK^-) are grown in standard culture medium and X-irradiated to stop cell division. The membrane properties of these cells can be studied by conventional intracellular electrophysiologic techniques under various culture conditions. Morphological changes produced by various agents are photographed with phase or Nomarski optics. Time lapse cinematography is used for a detailed analysis of the types of cellular movements involved in process formation. Standard techniques of electron microscopy are used to study the ultrastructure of irradiated L-cells treated with various agents. Movements of the surface membrane are followed by use of the extracellular tracers ruthenium red and horseradish peroxidase for varying lengths of time during process development and disappearance. The identification of sites of divalent ion binding or accumulation is being attempted by inclusion of multivalent ions (Ca, Sr, Co, ruthenium red) in the fixation solutions and by fixation in the presence of anions (phosphate, pyroantimonate) that precipitate cations of interest.

Major Findings: Time lapse films show that γ-irradiated L-cells undergo periodic changes in morphology in the normal culture medium. They alternate with time between flat polygons and spheres. These alterations probably correspond to phases of the cell cycle. The spherical cells in culture medium have a resting membrane potential of -15 to -20 mV and input resistances between 20 and 30 MΩ. These cells hyperpolarize in response to mechanical and electrical stimulation and to iontophoretically applied acetylcholine. During this "hyperpolarizing activation" (HA) response the membrane potential increases to a level of -35 to -45 mV and the membrane resistance decreases. The HA is produced by an increase in the membrane permeability to K^+. A similar increase in K^+ permeability can be produced by intracellular injection of Ca^{++}.

Electron microscopy reveals that the various morphological types of cells in untreated cultures have correspondingly different ultrastructures. An outstanding feature of the flat polygons is a radial arrangement of bundles of microtubules and microfilaments extending toward the points of the polygon. They also contain abundant cytoplasmic organelles. In contrast, the cytoplasm of the smooth surfaced spherical cells contains primarily single ribosomes and a few polyribosomes. In cultures treated with dibutyryl cAMP time lapse cinematography shows that process formation involves resculpturing of the cell body as well as growth of processes. The transformation can first be detected in flat polygonal cells in which areas between the points of the polygon retract leaving the points as the tips of the newly formed processes. The tips then elongate beyond the original limits of the cell. During these events the cell body becomes more spherical. The electron microscope shows that the processes contain microtubules and microfilaments which probably correspond to the radially arranged bundles that were present before treatment. The tips of processes, in general, resemble the growth cones at the tips of growing neurites. The cell body contains cytoplasmic organelles of the same sorts as the flattened polygonal cells. A Golgi apparatus usually is prominent near the base of each process. There are several populations of membrane bounded vesicles which are distinguished on the basis of size and associated coat materials. Studies with extracellular tracers suggest that one class of large vesicles may be formed by endocytosis during retraction of large areas of the cell surface which occurs as a part of the resculpturing of the cell. Another population of vesicles appears to be a source of new membrane to be inserted into the pre-existing membrane at sites of expansion.

The membrane properties of spherical cells with long processes as a result of db cAMP treatment differ from those of spherical cells in untreated cultures. They have a somewhat higher resting potential and a significantly lower input resistance due to a higher K^+ permeability. In these ways the membrane properties of the cAMP treated cell resemble the properties of the untreated cell during the hyperpolarizing activation response. Since the permeability properties of the cell during HA could be mimicked by intracellular injection of Ca it is of interest to explore the possibility that changes in intracellular Ca concentration or distribution are associated with the alterations in morphology. Certain alterations in divalent ion concentrations and composition of the culture medium can produce morphologic changes that resemble the process formation produced by cAMP. Preliminary results suggest that different areas of the cells have different cation-binding properties which may be related to sites where extension or retraction takes place.

The effect of cAMP on L cell morphology is reversed within 2 hours of removal of the drug. In the phase microscope the cells seem to return to their previous appearance. The typical organelles of the polygonal cells are still present, including the radially arranged microtubules and microfilaments. However, cells after "recovery" also contain large numbers of membrane bounded structures filled with cellular debris which suggests that autophagic activity plays a role in such morphologic changes.

Significance to Biomedical Research and the Program of the Institute: The interaction of cellular mechanisms underlying differentiation is one of the most fundamental questions in developmental biology. Until now a thorough analysis of the roles of the various cellular organelles in the restructuring of a non-differentiated cell into a cell with a new form and function has been lacking. At the same time much attention has been focused on the role of the cell membrane in coupling changes in the external environment with alterations in cellular machinery that are finally expressed as changes in morphology. The combination of electrophysiology which gives information about membrane properties with time lapse cinematography and electron-microscopy which provide insights concerning the formation, removal and re-distribution of cellular components has considerable promise in attacking these questions.

Proposed Course: We intend to follow in detail the turnover of surface membrane with extracellular tracers during the course of process formation and its reversal. The distribution of cation binding sites on the surface membrane will be analysed in relation to sites of membrane addition and removal during cell shape changes. The effects of agents that alter the membrane permeability to Ca and agents that alter the cell's ability to maintain ionic gradients by interfering with energy dependent ion pumps will be studied. We hope to develop a comprehensive model for the cellular activities involved in process formation and their regulation, and, finally, to test the model in experiments with differentiating neurons in cell culture.

Honors and Awards: None

Publications: None

NICHD ANNUAL REPORT
July 1, 1973 through June 30, 1974
Laboratory of Molecular Genetics

The Laboratory of Molecular Genetics conducts research directed toward understanding the molecular processes involved in the transmission of genetic information and in its regulated expression during growth and differentiation. A variety of model systems are under investigation, ranging from bacterial and animal viruses to complex vertebrate regulatory responses involving the synthesis of specific antibodies and the development of specific organ systems. Taken together, the laboratory joins active workers in the areas of genetics, biochemistry, immunology, virology and cellular biology to bring their skills to bear on problems of mutual interest. At present, the laboratory is comprised of nine research groups. Their activities are described in the phylogenetic order of the biological systems with which their research is primarily concerned.

A. Studies have been directed towards understanding the detailed mechanism of bacteriophage λ insertion and excision from its host chromosome, the regulated expression of phage genes, and the packaging and maturation of phage particles. A new and extremely powerful assay has been devised to isolate genetic mutations which are defective in their ability to excise from the host chromosome. This procedure has yielded a group of over 300 new mutants which will provide the basis for elucidating, genetically and biochemically, the mechanism of genetic integration and excision. In addition, the relationship between genes required for the recombination of host genetic information and viral genetic information has been studied, and the relationship between host and bacteriophage gene has been determined. In brief, recombination between genetic material of the bacteriophage and its host requires both bacterial and phage genes, whereas recombination between bacteriophage require only bacteriophage genes. The precise nature of these genes will be the subject of continuing studies.

Additional studies have been directed towards understanding the process of bacteriophage packaging and maturation. In brief, the packaging of bacteriophage DNA into its coat shell has been shown to be a polar process, depending upon at least one appropriate end structure in the DNA and further dependent upon enzymes capable of hydrolyzing DNA which is too large to fit into the phage capsule. Interestingly, while the genes necessary for the formation of the phage capsule can be involved either in providing structural elements or regulating or determining the specificity of capsule assembly, one of these products seems to play a role in regulating the synthesis of several viral capsular proteins. (Dr. Robert Weisberg, Principal Investigator)

B. Studies of the integrative control of macromolecular synthesis have focused on the role of the unusual nucleotides, pppGpp and ppGpp, which seem related to the coupled regulation of protein and nucleic acid biosynthesis. The studies during the past year have lead to an extensive purification of the protein [stringent factor (SF)] necessary for the biosynthesis of these unusual nucleotides and permitted detailed studies of the enzymatic reaction. While in general the reaction requires the presence of many of the elements necessary for protein biosynthesis, including ribosomes, uncharged tRNA and message, unusual conditions have been developed elsewhere and in this laboratory which permit the biosynthetic reaction to be studied using the stringent factor protein alone. This minimal reaction has facilitated the purification procedure as well as indicated that the active site for enzymatic catalysis resides on the SF protein itself. Studies of the degradation of these nucleotides, which together with the biosynthetic reaction determine the level in the cell, have indicated that the requirements are quite different from the biosynthetic reaction. For example, a complete array of aminoacylated transfer RNAs are necessary for degradation. In addition, studies directed towards understanding what regulatory affect these nucleotides might have on the transcription reaction have focused upon the reaction involving a binding between ppGpp and pppGpp and the enzyme RNA polymerase. This interaction has been studied using fluorimetric analysis and equilibrium dialysis techniques. These studies confirm that an interaction occurs between these nucleotides in the enzyme and suggest that a configurational change in the enzyme occurs. This may form a basis for the regulatory affect of these nucleotides upon the process of transcription.

The effect of ppGpp and pppGpp on the reactions involved in protein synthesis have also been studied. ppGpp can effectively substitute for the reactions involved in initiation and those catalyzed by the elongation factor T, however, the translocation reaction is not supported by ppGpp. Further studies have indicated that reactions generally associated with translocation do occur in the presence of this analog, suggesting that the inhibitory affect is at an unknown step in the translocation process, or that these reactions are not associated with true translocation. (Dr. Michael Cashel - Principal Investigator)

C. Factors which influence the rate of biosynthesis and level of accumulation of messenger RNA in cells have been studied with special reference to enzymes that are involved in the degradation and processing of messenger RNA. These studies have resulted in the purification of two ribonucleases which may play very important roles in the processing of messenger RNA and in the life cycle of specific viruses. Thus, ribonuclease III of E. coli, an enzyme which degrades double-stranded RNA, was shown to be separate and distinct from ribonuclease H, an enzyme which degrades RNA involved in RNA/DNA hybrids. Ribonuclease H has been found in all RNA tumor viruses investigated, an observation consistent with its presumed role in the life cycle of the virus. In addition, these enzymes have been identified in KB cells, chick embryo and E. coli and their purification will permit a detailed study of their mechanism of action and the elucidation of their functional roles. (Dr. Robert Crouch - Principal Investigator)

D. Studies directed towards understanding the regulation and expression of viral genes and the process of virus assembly have been carried out using a murine leukemia virus. A system has been developed which uncouples viral RNA synthetic reactions from the terminal steps in virus maturation. This system permits the observation of virus particle production in the absence of viral RNA biosynthesis and has led to the interesting conclusion that murine leukemia virus infected cells contain pools of structural elements which can be utilized for the terminal steps in assembly of viral particles. This occurs in the absence of ongoing viral RNA synthesis. Further, the viral particles which are released from uncoupled cells consist of two classes, 1) the standard virus, 2) defective virions which have several properties of leukemia virus but which are deficient in high molecular weight 70S RNA. Future studies will be directed towards understanding the role, if any, that viral RNA might play in the assembly of viral proteins and in understanding the structure of these defective particles. (Dr. Judith G. Levin - Principal Investigator)

E. Studies directed toward the identification of viral macromolecules and the description of their spacial and functional roles have been pursued using poliovirus, adenovirus and membrane-bound RNA oncogenic viruses as model systems. This research program has recently been added to the activities of the Laboratory of Molecular Genetics, and represents a continuation and extension of ongoing research programs previously conducted at the Albert Einstein College of Medicine in New York. Inasmuch as the laboratory has so recently been established at the National Institutes of Health, this summary represents both carry-over and current progress.

All proteins encoded by the RNA viral genome of polio have been identified and related to the structure of the polio virus. In addition, a more complicated animal cell DNA virus, adenovirus has been studied with similar objectives. The detailed architecture of the virion has been described and refinements of technique have revealed most, if not all, the protein accounted for by the viral genome. Interestingly, the distribution of viral protein products has now been assigned to either early, or late viral functions and the need for proteolytic cleavage of viral precursor proteins have been recognized. Despite the fact that the RNA tumor viruses have never been physically or chemically defined, initial work has led to the development of effective ways to purify such viruses based on their glycoprotein content. This technique, involving precipitation with the plant lectin, concanavalin A, should permit a detailed investigation of biologically active viral proteins. (Dr. Jacob V. Maizel, Jr. - Principal Investigator)

F. Studies have been directed towards establishing the identity and localization of specific adenovirus gene functions. These have led to the adaptation of a cell-free system capable of utilizing adenovirus Type 2 messenger RNA for cell-free protein synthesis. All the major structural proteins of the virion have been synthesized in vitro and mRNAs coding for various polypeptides have been partially fractionated with the aim of their eventual purification. Ultimately these studies should lead to the identification of specific adenovirus mRNAs which can be used to identify specific viral gene loci. (Dr. Heiner Westphal - Principal Investigator)

G. Studies have been directed towards understanding the sequence of events leading to the differentiation of lens fibers in the chick embryo. Prevously this group has developed a culture system, derived from an early embyronic chick lens epithelium, in which the normal morphological and biochemical aspects of lens fiber differentiation take place in vitro in a chemically defined environment. The investigations during the past year have concentrated on the mechanisms of specialization of protein synthesis and of cell elongation during the differentiation of lens fibers. The principal lens fiber protein, delta crystallin, was shown to have a molecular weight near 200,000 daltons and to consist of four 50,000 dalton subunits. Delta crystallin mRNA has been isolated from differentiating chick lens fibers; it has been definitively identified by translation in cell-free systems derived from Krebs II ascites cells and rabbit reticulocytes, and shown to have a molecular weight of 6 to 7 x 10^5. Parallel analyses of protein synthesis and mRNA profiles in differentiating lens fibers have indicated that the specialized synthesis of delta crystallin is accompanied by a correspondingly specialized mRNA content. Delta crystallin mRNA was demonstrated to be an efficient template for the avian myeloblastosis virus reverse transcriptase and the synthesis of a radioactive cDNA for delta crystallin will provide an effective tool for further study of the regulated expression of the delta crystallin genes. Since a method has been established to determine the absolute rate of protein synthesis in explanted lens epithelia, quantitation of delta crystallin mRNA by use of the cDNA probe will enable precise determination of the amount of transcriptional and translational control of delta crystallin synthesis during differentiation.

Studies concerning the mechanism of lens cell elongation have provided evidence against a role for cyclic AMP or cyclic GMP in this process. Furthermore, investigations of permeability suggest that influxes of macromolecular precursors or energy supplies are not essential for the initial phase of lens cell elongation. (Dr. Joram Piatigorsky - Principal Investigator)

H. Studies have been directed towards understanding the regulatory pathways affecting antibody biosynthesis at the cellular level. Specifically, much effort has been directed towards understanding the role of accessory cells (thymus influenced or derived T-cells). Several lines of evidence suggest that these T-cells may serve either to enhance or to suppress antibody secretion and proliferation of bone marrow derived cells (B-cells), the cell involved in the secretion of circulating antibody. In particular, the observation that streptococcal exotoxin (SPE) serves as a strong immunosuppressant and that, after treatment with this agent, recovering animals exhibited a very elevated antibody level, suggested that the effect of this protein exotoxin might be at the level of the T-cell. These observations have been extended to the mouse, an organism which permits the exploitation of an interesting genetic model involving the nude locus. These mice, which lack thymuses, provide an extraordinary model in which to test this hypothesis. Consistent with the postulation, SPE fails to suppress and does not enhance antibody production in athymic mice. Previously reported studies suggested that dibutyryl cyclic AMP exhibited a T-cell-like effect in thymectomized mice. This effect has been confirmed using cultured spleen cells from homozygous nude animals. (Dr. Edgar E. Hanna - Principal Investigator)

I. Studies have been directed towards understanding the regulated expression of mammalian genes. Two model systems have been chosen for this purpose, both derived from a hematopoietic organ. Earlier studies using erythroleukemic cells as models for erythrodifferentiation suggested that the regulation of globin gene expression occurred at the level of transcription. During the past year a series of phenotypic variant cloned cells have been obtained which differ from one another in the extent to which they can be induced to undergo erythrodifferentiation. Certain of these cells also differ in the amount of constitutive globin mRNA which they produce. Hybrid cell lines have been established in order to assess the dominance relationships that exist between these variant cell types. Preliminary studies indicate that the extent of erythrodifferentiation as measured by globin mRNA production is controlled by diffusible cell product which limits the accumulation of globin mRNA in hybric cells. Additional studies have indicated that leukemia virus shed from erythro-induced cells incorporate cellular mRNAs. The significance of this observation relates to the formal possibility that RNA tumor viruses may be used to convey genetic information from cell to cell. The result also influences the interpretation of considerable data obtained using the reverse transcripts of purified virus.

Considerable progress has been made in studies directed towards understanding the organization of antibody genes. A immunoglobulin mRNA corresponding to a mouse kappa light chain has been purified to virtual homogeneity. This mRNA serves as a template for the avian myeloblastosis virus reverse transcriptase. Synthetic DNA complementary to this message has been used to distinguish between several models advanced to explain the diversity of antibody structure. These studies clearly indicate that the genetic sequence corresponding to the constant region of the immunoglobulin light chain is represented very few, possibly less than 3 times, per haploid genome. These results rule out a stringent type of germ line hypothesis which has been advanced to explain the organization of immunoglobulin genes. They further require the postulation of a recombination step which - at the level of the gene or the message - would serve to bring together variable and constant regions of the light chain. Further studies using this approach should permit us to distinguish between the possibilities that antibody diversity has arisen through evolution or repeatedly arises during the somatic differentiation of immunocytes.
(Dr. Philip Leder - Principal Investigator)

Serial No. HD-LMG-5
1. Laboratory of Molecular Genetics
2.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Control Mechanisms in Temperate Bacteriophage λ

I. The mechanism of phage integration and excision
II. Packaging of viral and bacterial DNA by λ
III. General recombination by λ
IV. The physiology of phage that are unable to circularize their DNA
V. Regulation of gene expression in λ

Previous Serial Number:	HD-LB16
Principal Investigator:	R. Weisberg
Other Investigators:	N. Sternberg L. Enquist
Cooperating Units:	Laboratory of Molecular Biology, Section on Biochemical Genetics, NCI; Dr. K. Murray - Department of Molecular Biology, University of Edinburgh, Great Britain; Dr. D. Friedman - Department of Microbiology, University of Michigan, Ann Arbor, Mich.

Man Years

Total:	3
Professional:	3
Other:	0

Project Description:

Objectives: To understand the mechanism of the insertion of virus DNA into and its excision from the chromosome of its host; to study the nature of abnormal phage insertion and excision with the aims of understanding the normal process, on the one hand, and of determining the feasibility of using abnormal insertion as a tool for biochemical analysis of bacterial gene products on the other; to analyze the mechanism of conversion of the virus nucleic acid to a mature, infectious virus particle; to study regulation of λ gene expression.

Methods Employed: The organisms of study are bacteriophage λ, other genetically related phages, and their host, the bacterium *E. coli*. Genetic techniques are being used to obtain cells which carry virus DNA inserted at abnormal chromosomal sites; from such abnormal lysogens, we obtain novel types of transducing phage lines (phage strains whose chromosomes contain DNA of bacterial origin). Phage mutants with specific chromosomal aberrations are being used to characterize the activity and stability of the factors which govern insertion and excision. Temperature sensitive control mutants are being used to study the regulation of the synthesis of these factors. Density gradient analysis and electron-microscopic visualization of DNA are being used to determine the structure of the chromosome of a newly isolated phage variant. A bacterial mutant that is defective in one of the steps of virus morphogenesis is being used to characterize the cellular structures required for viral development. Likewise, morphogenesis-defective phage mutants are being used to determine the sequence of steps involved in the synthesis of the λ head.

Major Findings: (1) In the course of lysogenization by phage λ, the viral DNA is inserted into the bacterial chromosome at a specific chromosomal site called the attachment site. We have shown that insertion still occurs, although at reduced frequency, in mutant bacteria that have lost the attachment site. Such abnormal insertion can occur at many different chromosomal locations, although it otherwise resembles normal insertion. Occasionally, abnormal insertion of the virus DNA occurs within a bacterial gene, thereby inactivating the function of that gene. We previously found that insertion of viral DNA into a bacterial gene is a precisely reversible process. We know this because loss (or excision) of the viral DNA lead to restoration of gene function (see last year's report). Excision from within a gene requires the same virus-specific proteins that are required for excision of λ DNA from the normal attachment site: the *int* and *xis* proteins. From these results we have developed a powerful genetic selection for mutants in the *int* and *xis* genes. Plaques formed by mutant phage on hosts carrying a λ insertion in a bacterial gene can be easily distinguished from wild type plaques by the absence of revertant bacteria in the former case. We have isolated over 300 new *int* and *xis* mutants by this technique. We hope that characterization and mapping of these mutants will reveal: (a) whether *int* protein interacts with *xis* protein; and (b) whether the different biological functions of the *int* protein can be independently inactivated by mutation and, if so, how mutations inactivating different functions are arranged on the genetic map of the *int* gene. (2) We have studied the insertion specificity of specialized transducing phage isolated from lysogens carrying λ at secondary attachment sites. In no case are these phage identical to λ gal or λ bio, the transducing phage lines isolated from normal lysogens. This suggests that at least some secondary attachment sites differ from the primary attachment site by more than one nucleotide pair (3) The red genes of λ and the recA and recB genes of *E. coli* specify proteins that promote recombination. We have discovered that the functioning of the phage and bacterial proteins are interdependent in one condition and independent in another: Recombination between phage and host genes (measured by transduction) requires recA$^+$ function and either recB$^+$ or red$^+$ functions; Recombination between phage genomes (measured in phage crosses) is independent of recA$^+$ function provided the red$^+$ genes are present. We suggest the following explanation: λ specifies a protein that can replace recA$^+$ function. The putative protein is encoded by a late gene and is thus not synthesized during transduction. (4) Normally, λ particles are produced by cutting and packaging DNA that is in the form of oligomers of two or more genome units joined head to tail. In addition, to normal phage heads, efficient packaging appears to require the presence in the DNA of at least two copies of a specific nucleotide sequence called cos (for cohesive end site). We have been studying the production of phage after induction of a lysogen with an excision defective prophage. Such a system allows us to ask if DNA packaging and phage maturation can occur when the substrate DNA contains only one cos site. The results we've obtained argue that phage are produced under such conditions at 10% the normal efficiency, provided the lysate is treated with DNase. The latter enzyme is presumably required because packaged DNA, cut only at a single cos site, would be expected to stick out of the head and must be trimmed away before the phage tail can be added. In addition to such particles, the above lysate also contains particles that carry only host DNA. These latter particles are presumably formed by initiating packaging at sites that resemble phage cos sites (pseudo-cos sites) on the host chromosome, and are recognized by their ability to transduce a variety of host genes. Like the former phage, these transducing particles are only maturable in the presence of DNase.

Using the above system we've elucidated the following points about the packaging process: (1) Packaging is assymetric, proceeding from the left to the right cohesive end; (2) Of the various λ "head" proteins, only the product of gene A seems to affect the specificity of DNA packaging. This finding is consistent with the observation that gene A is the determinant of the ter or cos-site cutting function; and (3) The product of "head" gene D is required in less than normal amounts when the DNA to be packaged is smaller than that of λ. This agrees with the proposed role of D protein in stabilizing precursor head-DNA complexes. (5) The levels of mRNA made by the eight λ genes controlling viral head assembly are nearly the same, but the proteins made from these mRNA species are present in vastly differing amounts. Our results suggest a role for the product of "head" gene B in the regulation of its own synthesis and in the syntheses of "head" proteins W and C. This type of autoregulatory control could account for the differences observed in the levels of the various phage "head" proteins. (6) Lambda gene N controls the synthesis of a protein that increases the level of "early" phage mRNA synthesis. In order to better understand how this regulation occurs, we have isolated RNA polymerase mutations of the host that retard the growth of phage λ. The barrier to viral growth in these mutant hosts occurs at the level of RNA transcription and is overcome by phage mutations that allow the virus to grow in the absence of N function. When such an RNA polymerase mutation is recombined with Nus, another host mutation known to interfere with phage N function, the resultant double mutant is more defective for λ growth than either of its parents. These results argue that phage gene N protein, the host's RNA polymerase and the host's Nus protein interact in such a way as to permit proper phage transcription.

A second approach used to study the role of N protein in phage transcription has been to characterize the mode of action of two phage mutations-nin and byp-which impart to the virus an ability to grow in the absence of N function. These studies have shown that nin and byp do not affect the same site of N action, and probably act in different ways to alleviate the need for N protein.

Significance to Biomedical Research and the Program of the Institute: The insertion and excision of viral (and other episomal) DNA is known to change the physiology of the host organism. In addition, these viruses (and other episomal elements) provide an important mechanism of genetic transfer and exchange in the microbial world. Viruses promote genetic transfer because of their ability to convert host genes to an infectious form by packaging them inside viral structural proteins. It is highly likely that such a successful symbiotic relationship has an analogy in the relationship between certain animal viruses and the cells of higher organisms. In fact it is known that animal viruses can package the DNA of their hosts although the biological significance of this phenomenon is unknown. Moreover, an understanding of the control mechanisms operative in these easily studied microorganisms has obvious relevance to an understanding of regulatory elements in higher organisms. The ability to direct the insertion of phage DNA to predetermined locations on the host chromosome should prove to be an extremely useful and widely applicable method for isolating gene products and studying the control of bacterial gene expression.

Proposed Course of Project: We plan to characterize our new integration-excision mutants as described in Major Findings; We intend to look for mutants with an altered specificity of insertion; We plan to characterize the particles that are matured by DNase in order to test our hypothesis that the DNA protrudes from the head before nuclease treatment and to determine if the DNA end that is inside the head resembles one of the normal λ DNA ends; We plan to continue studies initiated several years ago to determine if a λ variant unable to circularize its DNA can initiate normal transcription and DNA synthesis; We plan to determine what λ function, if any, can substitute for bacterial $recA^+$ function.

Honors and Awards:

Invited speaker at the Roche Institute Symposium on Genetic Recombination May 1973

Session Chairman at the Workshop on Comparative Aspects of Regulation in Temperate Phages, Stockholm - June 1973

Publications:

1. Shimada, K., Weisberg, R., and Gottesman, M.: E. coli Mutants Produced by the Insertion of Bacteriophage λ DNA. Genetics 73: 81, 1973.
2. Shimada, K., Weisberg, R., and Gottesman, M.: Prophage Lambda at Unusual Chromosomal Locations. II. Mutations Induced by Bacteriophage Lambda in Escherichia coli K12. J. Mol. Biol. 80: 297, 1973.
3. Weisberg, R.A., and Sternberg, N.: Transduction of $recB^-$ hosts is promoted by λ red^+ function. Gatlinburg Symposium on Mechanisms in Recombination, 1974, in press.

Serial No. HD-LMG-1
1. Laboratory of Molecular Genetics
2.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Integrative Control of Macromolecular Synthesis

Previous Serial Number:	Same
Principal Investigator:	Michael Cashel, M.D., Ph.D.
Other Investigators:	Hendrick A. Raué, Ph.D. Ernest Hamel, M.D., Ph.D. Paul Shapshak, Ph.D.
Cooperating Units:	None

Man Years:

Total:	3.4
Professional:	3.4
Other:	0

Project Description:

Objectives: The broad objective of this project is to understand cellular regulatory changes to physiological changes in nutritional and environmental conditions. The ensuing coordinate adjustments of cellular activities allow an avoidance of cellular injury and adaptive transitions to renewed cellular growth. Our particular interest centers on the regulator nucleotides ppGpp and pppGpp which apparently function as "coarse tuning" regulators of major biosynthetic activities. These activities include ribosomal and transfer RNA synthesis, protein synthesis and phospholipid synthesis as well as several areas of intermediary metabolism. Our inquiry into the biological roles of these nucleotides concerns 1) biosynthesis and further metabolism of the nucleotides, 2) regulation of gene expression by the nucleotides and 3) regulation of cellular biosynthetic activities mediated by ppGpp and pppGpp.

Methods Employed: In addition to more classical molecular biochemical techniques continued development of solubility chromatography has occurred. This technique involves selective elution of insoluble membranes and proteins from solid matrices by gradient solubilization. Fluorescence parameters of RNA polymerase has been investigated under different conditions of gene expression. Continued work as centered on the derivation of analogs of nucleotides bearing either substituents at the 3' hydroxyl position or lacking the 3' hydroxyl group entirely; these analogs have been converted to other nucleotide derivatives enzymatically to yield an array of compounds useful for structure activity studies. Polyphosphate has been characterized in *E. coli* and a simple direct assay for the cellular content of this macromolecule has been devised.

Major Findings:

I. Biosynthesis and Further Metabolism of ppGpp

1. Characterization of the ribosomal dependent ppGpp synthetic reaction: As summarized in previous reports, ppGpp can be synthesized by an ATP mediated pyrophosphorylation of the 3' hydroxyl of ppG in a ribosomal dependent reaction. This reaction also has an absolute requirement at 37° for bound mRNA codons and codon-specific uncharged tRNA together with a protein now known as stringency factor (SF) protein. This protein has been purified at least 95% pure from salt washes of ribosomes; the ribosomal reaction it catalyzes is highly specific for the pyrophosphate donor (ATP) but uses both ppG and pppG as pyrophosphate acceptors. The acceptor function requires a 2' hydroxyl group (as well as a 3' hydroxyl group).

The activity of the SF protein is very likely specified by the gene that controls ppGpp accumulation in cells starved of amino acids since adding pure SF protein restores activity to otherwise inactive mutant ribosomes. This protein can bind stably to ribosomes from either mutant or normal cells. Early attempts to purify the mutant protein reveal no major discrepancies between the size or distribution of mutant SF protein suggesting a missense mutation. A suggestion of the functional defect in mutant SF protein comes from the observation that while normal SF protein requires about 1.5 moles uncharged tRNA mole ribosomes for 50% maximal ppGpp synthetic activity; mutant SF bearing ribosomal complexes require about 10 times more tRNA for equivalent activity. The reaction shows a tetracycline sensitivity in either case; this is notable since tetracycline inhibits both charaged and uncharged tRNA binding to the ribosomal acceptor (A) site and uncharged tRNA binding to the A site has been shown by workers at Harvard and Arrhus to be required for ppGpp synthesis on the ribosome.

2. Characterization of the minimal reaction: Sy and Lipmann have shown that crude preparation of SF protein can catalyze ppGpp synthesis without addition of ribosomes at low temperatures in the presence of 20% methanol. We have pursued this reaction in order to sort out attributes which are determined by the SF protein and those which are determined by the ribosomal complex. We find that mild detergents are far superior stimulants of this reaction than methanol. Apparently this requirement is dependent upon the hydrophobicity of the environment; activity can be obtained by binding the factor to hydrophobic ribosomes, membrane vesicles or by gentle detergent treatment. The reaction with the pure protein is sensitive to temperatures higher than 30° which result in inactivation of the enzyme. The substrate specificity of the ribosomal reaction is mimicked by the minimal reaction as is the tetracycline sensitivity; this indicates that these attributes are not ribosomal determined.

The minimal reaction has provided a simple means of assessing purification of SF protein which is much less susceptible to inhibition. Purification procedures by this criterion has led to increases of yield of SF protein at least 20-fold higher than procedures using the ribosomal complex assay. The protein is found both on low salt washed ribosomes and to a much greater extent on ribosome-free membrane vesicles. This partitioning of the protein is undoubtedly related to its hydrophobicity. The protein aggregates in low ionic strength buffers in the presence of multivalent cations and can be solubilized by high salt. A similar behavior is shown by many ribosomal 'structural' proteins and membrane proteins.

The minimal reaction is independent of mRNA or tRNA and shows about 10-fold weaker binding of its substrates. It seems likely that this reaction occurs in the cells for mutants unable to accumulate ppGpp during amino acid starvation none-the-less have basal levels of ppGpp which can slowly accumulate during carbon or nitrogen starvation when the degradation of ppGpp is markedly slowed.

3. Further metabolism of ppGpp: Recent work in a number of laboratories has led to deductions that while ppGpp is relatively stable in crude extracts (see previous report), the cellular lability of ppGpp is mediated by the phosphorylation of ppGpp to pppGpp which in turn is very labile. In vitro assays indicate that hydrolysis of pppGpp to ppGpp can occur by a variety of enzymes, but that hydrolysis of the 3' pyrophosphate residue of pppGpp has not been observed. Accordingly, we have utilized permeable cells to investigate this reaction; in such cellular preparations we have demonstrated that this mechanism exists as in whole cells with similar kinetics and regulation. The degradative reaction requires a complete array of aminoacylated tRNA species but is unaffected by protein synthesis inhibitors suggesting that a product is generated in consequence that is separate from the phenomenon of ppGpp synthesis. The reaction also requires (glycolytic) energy sources which have been localized to the level of triose phosphates by addition of glycolytic intermediates in place of glucose. The reaction is sensitive to detergents, which might explain the difficulties in observing the reaction in crude extracts as due to disrupting a heirarchy of cellular membrane ogranization. Efforts to understand the degrative reaction of pppGpp as well as the phosphorylation of ppGpp to pppGpp are in progress in both permeable cells and in partial reactions in extracts. We have characterized very major activities mediating nucleotide interconversions in permeable cells and find that polynucleotide phosphorylase plays a major role in this regard. Although most attributes of this enzyme behavior are identical in permeable cells to in vitro purified preparations, some interesting differences are notable in permeable cells.

II. Regulation of Gene Expression by ppGpp and pppGpp. The findings in cell-free systems coupling translation to transcription (in collaboration with the Zubay group) have indicated that gene expression can be either stimulated by ppGpp or inhibited. These effects are further highly specific for the structure of the added nucleotide and occur at the level of transcription rather than at the level of gene-specific translation (see last report). Accordingly, we have re-investigated the interaction of ppGpp with essentially pure RNA polymerase. In confirmation of our early studies (1970-71) we find binding of ppGpp and pppGpp to the enzyme by fluorimetric analysis and that this binding event is apparently specific to the initiation site of the enzyme. Binding of ppGpp is competitive with other initiation specific nucleotides and inhibited by an inhibitor of enzyme initiation, rifampicin. Confirmatory studies are in progress using equilibrium dialysis techniques. An additional finding is that an apparently new fluorescent peak is observed when initiation site binding occurs highly suggestive of a configurational change in the enzyme. Preliminary indications are that the enzyme might assume a different configuration in the elongation mode (in the process of elongating RNA transcripts) as compared to initial recognition of initiation sites on DNA. These observations rather strongly indicate that regulation of gene expression by ppGpp might well be exerted directly at the level of RNA polymerase-ppGpp interactions.

III. Regulation of Cellular Biosynthetic Activities by ppGpp and pppGpp.

1. Protein synthesis: The possibility that pppGpp plays a normal role in catalyzing the various reactions of protein synthesis has been explored. This possibility is raised by the fact that pppGpp and ppGpp accumulate when peptide bond formation is restricted as well by the dependence of protein synthetic reactions on guanine nucleotides. We have found that pppGpp can effectively substitute for GTP in reactions catalyzed by Initiation Factor 2 (IF-2) and also in the Elongation Factor T (EF-T) reaction. The pppGpp shows a contrasting inactivity towards the elongation reaction in protein synthesis catalyzed by Elongation Factor G (EF-G). This conclusion has been reached from studies of polyphenylalanine synthesis with a poly U as message. Attempts to localize the reason for inactivity of pppGpp in the elongation reaction revealed that the rate of EF-G dependent hydrolysis of pppGpp to ppGpp was largely intact; this occurs at about 30% the rate of GTP hydrolysis to GDP. Moreover, the rates of the fusidic acid dependent, EF-G dependent binding of pppGpp and ppGpp to ribosomes were about 75% and 85% the rates of GTP and GDP binding respectively. These partial reactions are either unrelated to protein chain elongation or represent a stage in elongation before the blocked step. Investigations of whether the inability of pppGpp to drive translocation can be attributed to the substituent on the 3' hydroxyl have been approached through the use of 3' analogs of GTP. These experiments indicate that such analogs are again less efficient in the elongation, but not as inactive as pppGpp. Attempts to localize the reason for inactivity of pppGpp in the translocation reaction even further are in progress. Inhibitor studies with ppGpp in comparison with GDP and GTP allows predictions of the effects of nucleotide pool changes during amino acid starvation on protein synthesis. The most susceptible reaction in protein synthesis is the initiation reaction under conditions of ppGpp at levels approaching GTP. Cellular studies with mutants showing a slow degradation of ppGpp when amino acids are restored (in other laboratories) have borne out this prediction: protein synthesis does not resume until ppGpp disappears from the nucleotide pools.

2. Polyphosphate synthesis: We have found that during amino acid starvation only one other phosphorylated species accompanies the accumulation of ppGpp and pppGpp. This is inorganic polyphosphate, a high molecular weight polymer with a high energy content. We have investigated the regulation of polyphosphate and found that the accumulation of polyphosphate follows the same regulatory rules as does the accumulation of ppGpp and pppGpp. Accordingly there is a possibility that ppGpp might be an intermediate in the formation of this energy reservoir in the cell under conditions where most cellular activities are restricted. Preliminary searches for the enzyme responsible for polyphosphate synthesis have led to the isolation of an ATP-dependent polyphosphate kinase, described by Kornberg in 1964. This enzyme has been purified about 1000-fold and the reaction appears to be unaffected by ppGpp or pppGpp as

measured both in the polymerization and the hydrolysis reactions. Radioactive pppGpp does not donate its gamma phosphate to form polyphosphate. Thus we are left being unable to account for the reason why mutants unable to accumulate have ATP dependent polyphosphate kinase activity nor does the polyphosphate kinase have SF protein activity.

Significance to Biomedical Research and the Program of the Institute: Integrative control processes in general, and adaptive regulation to nutritional and environmental changes in particular, are common to all growing cells. An understanding of these processes at any level contributes to how cells respond to their immediate environment and its changes. Amongst phylogenetically diverse bacteria the role of ppGpp has emerged as a master regulatory compound present in enormous amounts, controlled by perturbations in protein synthesis, capable of regulating genetic transcription and interacting with a variety of cellular functions that account for a sizeable part of cellular biosynthetic capacities. These include nucleotide synthesis, membrane transport, phospholipid synthesis, RNA synthesis, protein synthesis and glycolytic fermentation of sugars. Published hunting expeditions in other laboratories for the occurrence of ppGpp in eukaryotic cellular systems have so far met with limited success. The compound has been noted in differentiating slime mold and in plant chloroplasts, but not in plant cytoplasm. Adenine nucleotide analogs (pppApp and ppApp) have been reported to be synthesized by Actinomyces species. Our preliminary experience suggests approaches might be made with partially purified ribosomal systems in higher organisms because of potent inhibitory and degrative reactions. Very recently, ppGpp has been observed to be synthesized by a purified ribosomal system derived from wheat germ by Dr. A. Marcus. So far, searches for ppGpp from mammalian cells in culture have been uniformly negative despite the almost exactly parallel physiology when subjected to nutritional limitations.

Proposed Course of Project: Peptide maps of the protein catalyzing ppGpp synthesis will be prepared from normal and mutant bacteria as final proof that the enzyme is indeed the product of the RNA control gene. Further investigations will be made to attempt to relate the properties of this enzyme to the environment in which catalytic activities are manifested physiologically. Our studies on the mechanism of ppGpp modulation of gene transcription will be extended to include isolation of ribosomal and tRNA genes and their promotor fragments. Comparisons with genetic material whose expression seems regulated in cells by ppGpp might reveal interesting differences from genes not similarly regulated. Further efforts will be made to pinpoint the stage in protein synthesis elongation which recognizes the difference between pppG and pppGpp; this study may allow a further dissection of the mechanism of protein synthesis as well as produce information relating to regulatory nucleotides. Studies on the metabolism of ppGpp and pppGpp will be continued as indicated above.

Honors and Awards:

1. Invited Review: Annual Reviews Microbiology
   Fogarty Foreign Fellow Review Panel - 1974
   Chairman Ribosome Session: American Society Microbiology

2. Invited Speaker: Cold Spring Harbor Ribosome Meeting - September 1974
Johns Hopkins School of Medicine - January 1974
National Institute of Arthritis, Metabolic and Digestive Diseases - January 1974
University of Rochester School of Medicine - February 1974
Ribosome Session, American Society of Microbiology - May 1974
National Cancer Institute - June 1974

Publications:

1. Cashel, M.: Preparation of guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) from *Escherichia coli* ribosomes. *Analyt. Biochem.* 57: 100-107, 1974.
2. Cashel, M., and Gallant, J.: Cellular Regulation of ppGpp and pppGpp in *The Ribosome*. Cold Spring Harbor Press, 1974.
3. Hamel, E., and Cashel, M.: Role of guanine nucleotides in protein synthesis. Elongation Factor G and guanosine 5'-triphosphate, 3'-diphosphate. *Proc. Natl. Acad. Sci. U.S.* 70: 3250-3254, 1973.
4. Hamel, E., and Cashel, M.: Guanine nucleotides in protein synthesis. II. Utilization of pppGpp and dGTP by initiation factor 2 and elongation factor Tu. *Arch. Biochem. Biophys.* May 1974, in press.
5. Raue, H.A., and Cashel, M.: Regulation of RNA Synthesis in *Escherichia coli*. I. Characterization of cells subjected to simultaneous temperature and osmotic shock.: *Biochem. Biophys. Acta* 312: 722-736, 1973.
6. Raue, H.A., and Cashel, M.: Preparation of $[^{32}P]\beta$ labeled ribonucleoside 5'-triphosphates from permeabilized cells. *Analyt. Biochem.* 56: 129-136, 1973.
7. Raue, H.A., and Cashel, M.: Regulation of RNA Synthesis in *Escherichia coli*. II. Polynucleotide phosphorylase activity in cold-shocked cells. *Biochem. Biophys. Acta* 340: 40-51, 1974.
8. Raue, H.A., and Cashel, M.: Regulation of RNA Synthesis in *Escherichia coli*. III. Degradation of guanosine 5'-diphosphate 3'-diphosphate in cold-shocked cells. *Biochem. Biophys. Acta*, in press, 1974.
9. Que, L., Willie, G.R., Cashel, M., Bodley, J.W., and Gray, G.R.: Guanosine 5'-diphosphate, 3'-diphosphate: Assignment of structure by ^{14}C Nuclear Magnetic Resonance spectroscopy. *Proc. Natl. Acad. Sci. U.S.* 70: 2563-2566, 1973.
10. Yang, H.L., Zubay, G., Urm, E., Reiness, G., and Cashel, M.: Effects of guanosine tetraphosphate, guanosine pentaphosphate, and β-γ methylenyl-guanosine pentaphosphate on gene expression of *Escherichia coli* *in vitro*. *Proc. Natl. Acad. Sci. U.S.* 71: 63-67, 1974.

Serial No. HD-LMG-2
1. Laboratory of Molecular Genetics
2.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Factors Influencing Genetic Transcription-initiation and Termination

Previous Serial Number: Same

Principal Investigator: Robert J. Crouch

Other Investigators: Kyoko Uenoyama

Cooperating Units:
Dr. Takis Papas - National Cancer Institute, Viral Biology Branch
Dr. Vincent Hollis and Dr. Leo Phillips - National Cancer Institute, Viral Leukemia and Lymphoma Branch
Dr. Walter Keller - Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Man Years

Total:	1.4
Professional:	1.4
Other:	0

Project Description:

Objectives: RNA plays an important role in cellular regulation - either by its presence in active form or in an inactive form or by its total absence. A number of proteins have been shown to be involved in regulating RNA synthesis in *Escherichia coli* - among these are RNA polymerase, rho (or termination factor), kappa (a termination factor). These proteins are involved in the synthesis or inhibition of synthesis of specific species of RNA - and, as a result, control which RNA species are presented to the protein synthetic machinery of the cell. Although the demonstration of "inactive" messages has not been extensively considered in *E. coli*, recent results suggest that such a possibility is likely. Ribonuclease III (RNase III) is an enzyme from *E. coli* responsible for the processing of ribosomal RNA and the processing of RNA formed after infection of *E. coli* with bacteriophage T7. Certainly these results suggest that regulation of specific genes could occur by processing - or failing to process gene transcripts. Other RNA species may be regulated by another enzyme found in a wide variety of organisms; *E. coli*, KB cells, yeast, chick embryos, and calf thymus. This enzyme, RNase H, has the potential to regulate not only RNA synthesis but DNA synthesis. The objective of this research project is to determine the method by which several of these enzymes act to regulate RNA synthesis.

Methods Employed: Rho, kappa and what I call depression factor are all purified by standard techniques of ion exchange chromatography and sizing. RNase H assays are performed by degradation of ^{32}P labeled rA·dT and separation of the degradative products by thin layer electrophoresis on polyethyleneimine sheets. This technique allow us to distinguish between RNase H and other enzymes capable of attacking the substrate. A variety of acrylamide-gel electrophoretic techniques are also employed.

Major Findings: Previous work in other laboratories on RNase III indicated that this enzyme was able to degrade both double-stranded RNA and RNA of DNA-RNA hybrids producing RNA digests terminated with 3' phosphate moieties. Examination of these two properties in this laboratory revealed that a separate enzyme is responsible for the degradation of RNA of DNA-RNA hybrids and that the products of both enzymes are oligonucleotides with 5' phosphate termini. These results are critical for results of processing of T7 messenger RNA which show that both the *in vivo* and *in vitro* products of early T7 message processing can be accounted for by the action of RNase III.

Elucidation of the function of RNase H has not been completed but some steps have been made concerning the enzymatic activity and how complex the situation with this enzyme may be. First we have separated two RNase H activities from both *E. coli* and KB cells. A number of laboratories have suggested that RNase H is involved in DNA synthesis by virtue of the fact that RNA chains are responsible for initiation of DNA synthesis. A number of enzymes are capable of degrading this initiating RNA and, therefore, RNase H need not perform this function. Even if one of the RNase H activities that we observe is responsible for removal of initiating RNA, the function of the second is unclear.

Strong support that RNase H is involved in DNA synthesis comes from the observation that avian myeloblastosis virus (AMV) reverse transcriptase also possesses an RNase H activity. During the past year, I have examined a number of other tumor viruses for RNase H activity associated with their reverse transcriptases. RNA tumor viruses are notorious for being impure biochemically - and they often contain cellular components that can be demonstrated to be of cellular origin - not viral. All of the tumor viruses that we examined have RNase H activity. However, the ratio of reverse transcriptase to RNase H varies over 100-fold and the tumor viruses that were most highly purified has the least amount of RNase H compared to its reverse transcriptase activity. These results - along with those from other laboratories - suggest that for mammalian RNA tumor viruses - a majority of the RNase H which is observed is due to contamination with cellular RNase H. However, the RNase H one observes in AMV is associated with the reverse transcriptase. Isoelectric focusing columns of AMV extracts yields two peaks of reverse transcriptase activity. Although these have not been demonstrated to represent the $\alpha\beta$ and α composition of reverse transcriptase, it seems likely that these two peaks are of this composition. Nevertheless, we find that both of these peaks have RNase H activity.

Direction of Research in 1975: A continued examination of RNase H will be directed toward a clarification of the two RNase H activities found in *E. coli* and KB cells. Some indication that these are different enzymatically has already been observed. Also some attempts will be made to determine the function of RNase H by selection of mutants of *E. coli* which are defective in RNase H.

Studies concerning RNase III will focus on the ability of RNase III to process eukaryotic RNAs. This project will take two different approaches: 1) RNase III will be used to process RNA from eukaryotic organisms and 2) enzymes similar to RNase III will be studied from chick embryos to establish a relationship of recognition of RNAs between RNase III (from *E. coli*) and similar enzymes of eukaryotic organisms.

Significance to Biomedical Research and the Program of the Institute: The regulation of transcription can be essential in controlling gene expression in most organisms. Certainly changes in gene activity mediated through regulation of transcription can affect normal cellular activity while failure to regulate properly or to reorient transcription can be the basis of many medical abnormalities.

Honors and Awards:

1. Invited Lectures:
    a. Laboratory of Biochemical Pharmacology, NIAMDD
    b. Gordon Research Conference on Nucleic Acids, June 1974

Publications:

1. Crouch, R.J.: Ribonuclease III Does Not Degrade Deoxyribonucleic Acid: Ribonucleic Acid Hybrids. *J. Biol. Chem.* 249: 1314-1317, 1974.

Serial No. HD-LMG-8
1. Laboratory of Molecular Genetics
2.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Molecular Aspects of the Replication of Enveloped Animal RNA Viruses

Previous Serial Number: NCI-580
NCI-587

Principal Investigator: Judith G. Levin

Other Investigators: None

Cooperating Units: Dr. Philip M. Grimley - Division of Laboratories and Research, New York State Department of Health, Albany, New York;
Dr. Wallace Rowe - Laboratory of Viral Diseases National Institute of Allergy and Infectious Diseases

Man Years:

Total: 1-1/2
Professional: 1-1/2
Other: 0

Project Description:

Objectives: To study factors which influence expression of viral genetic information; to define in molecular terms the specificity requirements of the viral assembly process; to understand the virus-host relationship in chronic infection.

Methods Employed: Mouse cells chronically infected with murine leukemia virus are maintained as continuous lines in culture. Leukemia virus production is measured by assay of the extracellular fluids for reverse transcriptase activity in response to a synthetic template. Infectivity is determined by plaque assay. To characterize the RNA species present in virions. RNA is extracted from particles purified by sucrose density centrifugation and is then analyzed by polyacrylamide gel electrophoresis. Conditions have been devised which yield high resolution of a wide range of RNA molecules--from the high molecular weight 70S viral RNA genome to the low molecular weight 8S and 4S RNA species. Electron microscopy is used to follow viral morphogenesis and to delineate the ultrastructural features of the virions. Thin section electron microscopy and negative staining techniques have been employed.

Major Findings:

1. A system has been developed in which viral synthetic reactions are uncoupled from terminal steps in virus maturation. Mouse cells chronically infected with murine leukemia virus were treated with actinomycin D at concentrations (up to 5 µg/ml) which severely inhibit cellular and viral RNA synthesis. Nevertheless, virus production, as evidenced by the appearance of budding C-type particles and the presence of reverse transcriptase activity in culture fluids, continued for as long as 6-12 hours.

2. Virus particles released during 6 hours incubation with actinomycin D (1 µg/ml) had the ultrastructural morphology of typical C-type virions and sedimented in sucrose gradients with a buoyant density of 1.16 g/cm^3.

3. Assay of reverse transcriptase activity revealed a marked preference for poly rA·oligo(dT)$_{(12-18)}$ consistent with the presence of a viral RNA-dependent DNA polymerase. The magnitude of the polymer response was in the range of untreated controls. In contrast, ribonuclease-sensitive endogenous activity was 80% lower than control levels. This observation correlated with a 30-50-fold reduction in infectivity.

4. Kinetic studies on the appearance of labeled RNA in banded virions showed that within 1 hour after addition of actinomycin D, particles contained 70S RNA and some 8S and 4S RNA. After further incubation, 70S RNA was not detected and 4S RNA was the major species.

5. We draw several conclusions from these results:
    a. Murine leukemia virus-infected cells contain pools of virus-specific precursor molecules which can be utilized for terminal steps in assembly in the absence of ongoing viral RNA synthesis;
    b. At least two classes of murine leukemia virus particles are present in a 0-6 hour harvest from actinomycin D-treated cells: (1) standard virions and (2) defective virions which have several properties of standard leukemia virus but which are deficient in high molecular weight 70S RNA;
    c. The reverse transcriptase enzyme can be packaged within a C-type virion in the absence of its natural 70S RNA template.

6. In other related studies, it has been found that the murine leukemia virus-infected cells can be superinfected with a non-oncogenic RNA virus (Semliki Forest virus, one of the group A arboviruses). Preliminary evidence suggests that a small proportion of the progeny consists of hybrid virus particles (viral pseudotypes).

7. Arbovirus replication is not affected by the presence of endogenous murine luekemia virus in the host cell.

Significance to Biomedical Research and the Program of the Institute: The advantages of viral systems as models for studying the regulation and expression of genetic information are well recognized. Studies on the RNA tumor viruses are of special interest since these viruses establish a chronic infection in the host cell, presumably by integration of viral genetic information into the host chromosome. An understanding of the various steps involved in tumor virus replication and mechanisms for generating defective virions should aid in efforts to limit the oncogenic potential of these agents. Finally, information on the degree to which host material can be assembled into virus particles could have some application in the field of gene therapy, where the use of pseudotype virions has been contemplated as a means of introducing "foreign" nucleic acid into an individual possessing an inherited biochemical defect.

Proposed Course of Project: In the coming year, the characteristics of defective murine leukemia virus particles from actinomycin D-treated cells will be studied in greater detail and compared with the properties of standard virions from untreated cells. Several approaches including hybridization experiments will be used to determine whether the RNA contained in the defective particles is derived entirely from the host or whether some of it is virus-specific. This point is of some interest as it is not known whether the presence of viral RNA is required to direct the assembly of viral proteins into a recognizable leukemia virus particle. In addition, attempts will be made to examine more closely the properties of the reverse transcriptase enzyme in the defective virions and to analyze the protein content of these particles. In future work it is hoped that further studies on mixed arbovirus-murine leukemia virus infection can be pursued.

Honors and Awards:

1. Invited Lectures - National Institute of Arthritis, Metabolic and Digestive Diseases Seminar - February 1974; National Cancer Institute Seminar - May 1974
2. Chairman and Speaker, Session on Oncogenic Viruses, American Society of Biological Chemists, Annual Meeting, Minneapolis, Minnesota, June 1974.
3. Reviewer, Chemistry and Molecular Carcinogenesis Segment, Carcinogenesis Program, National Cancer Institute.

Publications:

1. Levin, J.G., Ramseur, J.M., and Grimley, P.M.: Host effect on arbovirus replication: appearance of defective interfering particles in murine cells. J. Virol. 12: 1401-1406, 1973.
2. Levin, J.G., Grimley, P.M., Ramseur, J.M., and Berezesky, I.K.: Deficiency of 60-70S RNA in murine leukemia virus particles assembled in cells treated with actinomycin D. J. Virol., in press.

Project No. Z01 MG-[illegible]
1. Laboratory of Molecular Genetics
2.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Morphogenesis of Animal Viruses During Infection of Mammalian Cells

Previous Serial Number: None

Principal Investigator: Jacob V. Maizel, Jr.
Margaret Stewart

Other Investigators: Jürg Meyer

Collaborating Investigators: Aya Leder

Cooperating Units: Dr. D. F. Summers - Albert Einstein College of Medicine, Bronx, New York

Man Years:

Total: 1.5
Professional: 1.5
Other: 0

Project Description:

Objectives: To identify the macromolecules produced by virus infection of human cells and describe their spatial and functional roles in replication of the virus and regulation of the host cell.

Methods Employed: Techniques for sensitive biochemical and biophysical characterization of proteins and nucleic acids are central to these studies. Electrophoretic methods have been developed permitting separation of 50-100 different sizes of protein or nucleic acid molecules and estimation of their amounts, sizes and number. Electron microscopic techniques are employed to characterize the 3-dimensional organization of proteins and nucleic acids in viruses and cell organelles, and a primary objective is to extend the usefulness of electron microscopy for macromolecular characterization of proteins and nucleic acids. Viruses representing widely different groups are used in conjunction with continuous mammalian cell culture to produce simplified and manageable mammalian systems patterned after molecular biological work with microbial systems.

Major Findings: This project represents a continuation of a program that was conducted for twelve years by the principal investigator at the Albert Einstein College of Medicine in New York. Initial work at the NIH has been limited in scope until laboratory facilities and equipment are fully operational and is described subsequent to a brief resume of previous accomplishments.

Three groups of mammalian viruses have been studied.

1. Poliovirus replicating in HeLa cells was the first system to attain the goal of the detection and analysis of all proteins encoded by a RNA viral genome. Polio was also the first virus to reveal involvement of proteolytic cleavage.
2. Adenovirus proteins have been studied with similar objectives to polioviruses (i.e., structural and functional analysis) but with the outlook of a DNA genome with approximately ten times the complexity of poliovirus. The detailed architecture of the virion (virus particle) proteins has been described and further refinements of technique have revealed most, if not all, of the proteins accounted for by the genome. The distribution of proteins within subcellular fractions has been preliminarily determined, and assignment of polypeptides as either "early" (before viral DNA replication) or "late" (after DNA replication) has been made. Proteolytic cleavage has been recognized as an intimate process in viral maturation, as with poliovirus.
3. Studies on membrane-bounded, RNA-oncogenic viruses has begun as a logical extension of previous virus studies and as an even more demanding challenge to techniques. These viruses were never physically and chemically defined and initial work has led to development of more gentle and effective ways to purify such viruses based on their glycoprotein content and its affinity for the plant lectin, concanavalin A (Con A). Use of Con A has enabled rapid analysis of virus producing cells leading to detection and resolution of different proteins between spontaneously arising endogenous murine oncornaviruses and Friend leukemia virus complex.

Since arrival at the Laboratory of Molecular Genetics, work has been completed on 3 publications related to the oncornaviruses, the con A purification and analysis has been performed (in collaboration with A. Leder) on a continuous murine cell line (derived by Ikawa) possessing certain Friend virus disease properties. Methods for electron microscopic analysis of RNA are being set up, and initial data on ribosomal, $Q\beta$ phase, murine oncornavirus, and messenger RNA's is being produced.

Significance to Biomedical Research and the Program of the Institute: Virus replication serves as a model for cellular processes, and consequently study of the relatively simple human, and other, mammalian viruses is revealing the basic processes of replication, assembly and regulation of human cells (and presumably the organism). Development of techniques needed for the special rigorous demands of these viruses has provided tools useful to almost all other biological systems (a case in point being the SDS-acrylamide gel electrophoresis system first employed with adenovirus and poliovirus). These techniques and methodological approaches are directly applicable to studies on human embryonic development, infection, oncogenesis, and aging.

Proposed Course of Project: Some of the same viruses, cells and techniques will continue to be employed with special emphasis on the electron microscope detection of the genetic structure of polioviruses, oncornaviruses and adenovirus, the introduction of techniques to the laboratory for visualizing the structure of functioning poliovirus replicase, and intracellular localization of adenovirus proteins. Attempts will also be made to develop methods for obtaining sequence-related information from proteins along the lines of those used for nucleic acids.

Honors and Awards:

Invited lectures (since January 1)
  University of Maryland, March 1974
  University of Pittsburgh, May 1974
  Cold Spring Harbor Course on Animal Viruses, June 1974

Publications: None

Serial No. HD-LMG-4
1. Laboratory of Molecular Genetics
2.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Study of Adenovirus Gene Functions

Previous Serial Number: Same

Principal Investigator: Heiner Westphal

Other Investigators:
Lawrence Eron
Robert Callahan
Franz-Josef Ferdinand

Cooperating Units: None

Man Years

Total: 4.9
Professional: 3.9
Other: 1.0

Project Description:

Objectives: Study of molecular correlates of adenovirus gene functions.

Methods Employed: Adenovirus messenger RNA is prepared from virus-infected KB cells grown in cell culture. Messenger RNA is purified by adsorption to oligo(dT)-cellulose which selects for poly(A)-containing RNA sequences. Some RNA preparations are further purified by reversible hybridization with viral DNA. The coding specificity of messenger RNA is measured in a cell-free protein-making system derived from mouse Ehrlich Ascites tumor cells. In vitro synthesized proteins are identified by size, primary structure and antigenicity, using authentic virion proteins as a reference.

Major Findings: Pure adenovirus type 2 messenger RNA was obtained from infected cells and translated in the cell-free protein-making system. All major structural proteins of the virion were synthesized in vitro. Messenger RNA's coding for the various polypeptides could be partially fractionated by velocity centrifugation through sucrose gradients.

Significance to Biomedical Research and Program of the Institute: With the in vitro synthesis of adenovrius proteins, conditions have been created for a thorough analysis of the gene functions of this tumor virus. It is expected that this analysis will lead to an understanding, on the molecular level, of the expression of virus genes in the infected cell.

Proposed Course of Project:
(1) Mapping of adenovirus type 2 gene functions. Messenger RNA will be annealed to specific fragments of the DNA. This procedure will establish populations of RNA with distinct primary structure. Translation of the RNA's will enable us to determine gene functions encoded in specific portions of the genome.
(2) Early viral messenger RNA will be translated in vitro in order to obtain the molecular correlates for gene functions such as those needed for viral DNA replication and for the alteration of cell growth patterns. These functions will be mapped as indicated above. An attempt will be made to test these functions in vivo by infecting cells with selected genome fragments carrying early genes.

Honors and Awards: None

Publications:

1. Eron, L., Callahan, R., and Westphal, H.: Cell-free Synthesis of Adenovirus Coat Proteins. J. Biol. Chem, accepted.

Serial No. HD-LMG-3
1. Laboratory of Molecular Genetics
2.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Molecular Aspects of the Differentiation of Embryonic Chick Lens Fibers *in vivo* and in Tissue Culture

Previous Serial Number: Same

Principal Investigator: Joram Piatigorsky

Other Investigators:
Peggy Zelenka
Leonard M. Milstone
David C. Beebe
James C. Liang (Visiting Student)

Cooperating Units:
Gerald W. Robison - National Eye Institute;
Gerald J. Chader - National Eye Institute;
Robert T. Simpson - National Institute of Arthritis, Metabolism and Digestive Diseases;
Laboratory of Vision Research, National Eye Institute;
Laboratory of Nutrition and Endocrinology, National Institute of Arthritis, Metabolism and Digestive Diseases

Man Years

Total:	4.8
Professional:	4.3
Other:	0.5

Project Description:

Objectives: The objective of this research is to describe in detail the sequence of events leading to the normal differentiation of lens fibers and to understand the regulatory mechanisms at the molecular level. The lens fiber is chosen as a model system to study cell differentiation for 2 reasons. First, it involves marked changes in morphology (cell elongation and nuclear degeneration) and synthesis of lens specific protein (80% of the cell protein is delta crystallin). Secondly, the process of lens fiber differentiation takes place in tissue culture and thus is amenable to experimental analysis.

Methods Employed: Histology was performed by conventional procedures using Carnoy's fixation and paraffin embedding; electron microscopy was done using glutaraldehyde fixation and Epon embedding. Cell length was measured from histological sections using a calibrated ocular micrometer. mRNA was phenol extracted from fractionated homogenates of lens fibers of 15-day old chick embryos; it was purified by oligo(dT)-cellulose chromatography. The mRNA was analyzed electrophoretically and its activity tested in cell-free protein synthesizing systems. Delta crystallin was purified by isoelectric focusing and studied by electrophoresis, ultracentrifugation and amino acid analysis. Other methods employed involved conventional biochemical techniques, mostly utilizing radioactively labeled materials.

Major Findings:
1. The molecular weight and subunit structure of delta crystallin were shown to be different from that reported by others. The molecular weight of purified native delta crystallin obtained from lens fibers of 13- to 17-day-old chick embryos was determined to be approximately 200,000 daltons by gel filtration and sedimentation equilibrium centrifugation. The subunit molecular weight of delta crystallin was found to be between 45,000 and 50,000 daltons when examined in the presence of sodium dodecyl sulfate by electrophoresis and at sedimentation equilibrium. Delta crystallin was completely dissociated by sodium dodecyl sulfate without reduction, indicating that the subunits are not covalently bound by disulfide bridges. Thus, native delta crystallin appears to consist of four polypeptides of the same size.

Delta crystallin was heterogeneous in sedimentation and electrophoretic experiments in the presence of 8 M urea or 5 M guanidine hydrochloride. This finding requires that previous experiments with urea concerned with the subunit composition of delta crystallin be re-examined.

Amino acid composition analyses showed that delta crystallin has an unusually high proportion of leucine and isoleucine, namely 15% and 7.5%, respectively. Possibly the high leucine and isoleucine content contributes to the stability of delta crystallin to urea and guanidine hydrochloride.

2. Delta crystallin mRNA has been isolated, partially characterized, translated in vitro and reverse transcribed. Seventy to 80% of the protein synthesized and accumulated during differentiation of embryonic chick lens fibers is the tissue specific protein delta crystallin. We have isolated and partially characterized the total cytoplasmic mRNA from purified lens fibers of 15-day-old embryos as an initial step toward understanding the regulated expression of delta crystallin during development. Each lens fiber mass contained an average of 10 μg of cytoplasmic RNA; approximately 0.1 μg per fiber mass was recovered in the mRNA fraction by oligo(dT)-cellulose chromatography. The mRNA electrophoresed primarily as a single peak on a polyacrylamide-agarose gel with an apparent molecular weight of about $9x10^5$ estimated by comparison with 28S and 18S rRNA markers. Analysis by formamide gel electrophoresis indicates, however, that this is an overestimate and that the molecular weight of delta crystallin mRNA is $6-7x10^5$. Seventy to 80% of the protein synthesized in response to the mRNA in cell-free systems derived from Krebs II ascites tumor cells or rabbit reticulocytes co-migrated with delta crystallin on SDS-polyacrylamide-agarose gels. Comparison of the tryptic peptides of delta crystallin with those of the in vitro products from both heterologous systems, established that the lens

fiber mRNA contained delta crystallin mRNA, and that no other functional mRNAs were present in detectable quantities. Thus, the specialization of protein synthesis in embryonic chick lens fibers apparently results from an accumulation of delta crystallin mRNA in the cytoplasm.

After purification by sucrose density gradient centrifugation, delta crystallin mRNA has been reverse transcribed with AMV reverse transcriptase. The cDNA obtained appears quite large (~6S) but not as large as the complete mRNA (17-18S). Hybridization experiments suggest that the mRNA is reasonably pure, although small amounts of 18S rRNA may still contaminate the preparation.

3. The absolute rate of protein synthesis has been determined for the explanted lens epithelial cells during the first day of cultivation. As an initial step in determining precisely the amount of transcriptional control and the amount of translational control during differentiation of the lens cells, the absolute rates of synthesis have been measured for total protein and for delta crystallin during the first 24 hours of cultivation. The calculation of these values depends upon knowledge of the intracellular specific activity of the amino acid used for making the determinations. We have adapted a double label, 2 dimensional chromatography method using dansylated amino acids in order to determine the specific activity of the intracellular H^3-valine pool. Surprisingly, and to our advantage, the intracellular specific activity of H^3-valine changed at an equal rate and achieved comparable values in epithelia labeled immediately after explantation, after 5 hours of culture or after 24 hours of culture. Thus, direct comparisons of rates of incorporation could be made between explants at these different stages of lens fiber differentiation. After correcting for small changes in degradation of protein, it was calculated that total protein synthesis in each cell increased 1.4-fold while delta crystallin synthesis increased 3-fold within the first day of differentiation. Thus, the cultured lens epithelial cells appreciably increase their specialization of protein synthesis as do intact, developing lens cells.

4. Fetal calf serum and insulin were shown to stimulate modestly α-aminoisobutyric acid and uridine uptake, but not 2-deoxyglucose uptake, in explanted embryonic lens epithelia. The effects of porcine insulin (4 μg/ml) and fetal calf serum (15%) on the uptake of 2-deoxy-D-glucose $[^3H(G)]$ and α-aminoisobutyric-1-^{14}C acid by explanted 6-day-old embryonic chick lens epithelia were studied during the first 2 hours of culture. Insulin had no effect on 2-deoxy-D-glucose uptake while serum depressed uptake by 19%. About 60% of the accumulated label co-migrated with 2-deoxy-D-glucose-6-phosphate in thin layer ion-exchange chromatograms; the remaining radioactivity was found co-migrating at the front with 2-deoxy-D-glucose. By contrast, insulin and serum both stimulated uridine uptake by about 60%. About 90% of the accumulated radioactivity was soluble in trichloroacetic acid. The distribution of the nucleotide uptake product was the same in serum or insulin as judged by thin layer ion-exchange chromatography (35% UTP, 15% UDP, 25% UMP, 10% cytidine nucleotides). This stimulation was not abolished by inhibiting RNA synthesis with actinomycin D. The uptake of a nonmetabolizable amino acid, α-aminoisobutyric acid, was increased 30% by insulin and 88% by serum. Concentration studies in unstimulated epithelia indicated that the accumulation of each of the 3 com-

pounds tested was not limited by the number of available sites for uptake, suggesting that stimulation is due to greater efficiency of each site.

5. Experiments have suggested that cyclic AMP is not involved in promoting lens cell elongation *in vitro*. Numerous experiments have been conducted to test if cyclic AMP antagonizes the ability of insulin or serum to stimulate lens cell elongation *in vitro*, since current experiments in the literature suggest that both insulin and serum affect cells by lowering the intracellular concentration of cyclic AMP. Neither cyclic AMP (3 mM) nor dibutyryl cyclic AMP (1 mM) inhibited cell elongation promoted by serum or insulin. The combination of dibutyryl cyclic AMP and theophylline (1 mM) or papaverine (0.1 mM), which both inhibited cellular phosphodiesterase activity, also did not prevent elongation, although theophylline may have had a slightly toxic affect. Longitudinally oriented microtubules were present in cells treated with these compounds as they were in control explants. These data suggest that insulin and serum stimulate lens cell elongation by mechanisms not involving cyclic AMP. Initial attempts to determine other possible intracellular mediators of insulin or serum action have been unsuccessful. The most interesting candidate, which appears to have been eliminated is cyclic GMP, although caution must be exercised in interpreting negative experiments.

Significance to Biomedical Research and the Program of the Institute: One of the difficulties of studying cellular differentiation is that embryonic systems have many unidentified variables and generally involve interaction of many cell types. The differentiation of lens fibers has the advantages that (1) it occurs synchronously in a single population of excised epithelia cells, (2) all processes examined in culture mimic those which take place *in vivo* during the normal development of lens fibers, (3) large amounts of easily identified lens specific proteins are involved and (4) simple morphogenetic changes can be related to biochemical events. These studies, then, give information on the mechanisms of cellular differentiation, which is relevant to the goals of this Institute.

Proposed Course of Project: During the next year, the following projects will be completed or undertaken. (1) Delta crystallin cDNA will be characterized and used to quantitate the delta crystallin mRNA levels and delta crystallin DNA dosages in differentiating lens cells *in vivo* and *in vitro*. (2) Alpha crystallin mRNA will be isolated from calf lens and reversed transcribed; the homology of its cDNA with chick alpha crystallin mRNA will be tested, and if possible, the calf cDNA for alpha crystallin will be used to quantitate alpha crystallin mRNA in the differentiating chick lens cells. (3) The absolute rate of microtubule protein synthesis will be established at several stages of lens cell differentiation, (4) the pattern of protein synthesis will be determined in lens cells stimulated to divide rather than differentiate. The basis for the expected differences will be related to specific mRNA levels. (5) The number of different polypeptide chains composing native delta crystallin will be established. (6) Experiments will be continued attempting to determine unequivocally whether or not cyclic AMP is involved in insulin or serum stimulation of the cultured lens cells. (7) The possible role of the lens capsule in promoting epithelial cell elongation will be explored.

Honors and Awards:

1. Lectures (Joram Piatigorsky, except where specified)
    a. Selected speaker at American Society for Cell Biology, November 1973 (Peggy Zelenka).
    b. National Eye Institute Seminar, December 1973.
    c. New York Academy of Sciences Speaker for Conference on Microtubules, May 1974.
    d. International Meeting on Eye Research Speaker, Capri, Italy, June 1974.
2. Appointed Chairman, Department of Biology and Genetics, Foundation for the Advancement of Education in the Sciences, March 1974.

Publications:

1. Piatigorsky, J., Rothschild, S.S., and Milstone, L.M.: Differentiation of lens fibers in explanted embryonic chick lens epithelia. *Develop. Biol.* 34: 334-345, 1973.
2. Piatigorsky, J., Zelenka, P., and Simpson, R.T.: Molecular weight and subunit structure of delta-crystallin from embryonic chick lens fibers. *Exp. Eye Res.* *18*, in press.
3. Zelenka, P., and Piatigorsky, J.: Isolation and *in vitro* translation of δ-crystallin mRNA from embryonic chick lens fibers. *Proc. Natl. Acad. Sci. U.S.*, in press.
4. Piatigorsky, J.: *In vitro* lens cell elongation and microtubules. Annals New York Academy of Sciences, in press.

Serial No. HD-LMG-7
1. Laboratory of Molecular Genetics

3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Regulation of Antibody Biosynthesis
I. Mechanisms of consignment of cells to determinant-specific immunocompetency
II. Promoters and amplifers of antibody biosynthesis in cell culture
III. Initiation of determinant specific antibody biosynthesis in cell culture
IV. Deviation of immunocompetent cells

Previous Serial Number: HD-LB20

Principal Investigator: E.E. Hanna

Other Investigators: None

Cooperating Units: None

Man Years

Total: 12/12
Professional: 12/12
Other: 0

Project Description:

Objectives: To explore and delineate mechanisms for regulating pathways of antibody biosynthesis at the cellular level. Currently much of our effort is devoted toward understanding the roles of accessory cells (thymus influenced or derived T-cells). These cells appear to regulate the antibody secretions of bone marrow derived cells (B-cells). Regulatory effects of T-cells on the B-cell may be positive (enhancement) or negative (suppression). Whether both of these effects can be properties of the same T-cell population at different developmental stages during differentiation or whether two different subpopulations are responsible is not yet understood. The accessory role of macrophages in these processes is not yet understood but its importance is recognized by most investigators. We will test for any meaningful direct association between macrophages, T-cells and B-cells in our experiments.

We are pursuing an understanding of enhancement and suppression in lymphoid cell culture. With respect to enhancement, a helper T-cell effect, we have obtained good results with dibutyryl cyclic AMP in mice rendered T-cell deficient by thymectomy. Whether this nucleotide or similar compounds are active moieties of a possible "lymphokine" product of helper T-cells remains to be determined.

Our early observation in rabbits that a streptococcal exotoxin (SPE) was a strong immunosuppressant, and that animals recovering from suppression often exhibited greatly elevated antibody levels, lead us to postulate that SPE affects suppressor T-cells preferentially. We have recently found a similar response to SPE in a mouse model system (in vivo and in cell culture). The availability of athymic nu/nu (nude) mice and phenotypically normal +/nu littermates has greatly accelerated our current work toward a proof of our speculation that SPE may relieve B-cells from T-cell regulation. The ability to supplement athymic (nu/nu) spleen cell cultures with +/nu cells gives us great new ability to study these pathways more definitively. Mitogens such as endotoxins (LPS) of gram negative bacteria which selectively stimulate B-cell function, and others such as concanavalin-A and phytohemagglutinin which appear to stimulate the helper T-cell function preferentially and now SPE which appears to preferentially block the suppressor T-cell effect gives us great advantage in these studies.

Hopefully, as these exciting experiments continue to progress we may obtain useful means of regulating the protective immune response to human disease.

Methods Employed: Antibody secreting cells (mature B-cells) are detected as a result of discrete holes (plaques), formed in a confluent lawn of erythrocytes or erythrocytes bearing chemically linked defined determinants. Mitogens are used as described above. B-cell precursors may be detected by adherence (rosette) test very early using cells from immunized animals. The use of this assay may also assess T-cells when employed under some conditions with non-immune cells. The cells may be isolated on ficoll-hypaque gradients.

We rely upon our replica assay for direct assay of specificity of antibodies secreted by B-cells. Our ability to electronically rate-freeze cells, store them in liquid nitrogen, and recover essentially full biologic activity is convenient since it allows repeat-studies at will.

Major Findings: The in vivo helper T-cell-like effect of dibutyryl cAMP was published (1). We have recently found preliminarily that this compound will also stimulate nu/nu spleen cells in culture. This strongly supports our speculations set forth in this report.

SPE was found to suppress the plaque-forming cell (PFC) antibody response in mice and in culture of mouse spleen cells. The escape from suppression which is followed at 10-15 days by a greatly elevated response, as previously reported (2), is also obtained. Fully consistent with our speculation that SPE effects suppressor T-cells preferentially is that this effect is not observed in culture, nor in vivo using nu/nu athymic mice, but is obtained fully as described using +/nu heterozygoates. There are, however, alternative interpretations which we are proceeding to eliminate sequentially.

We have found in our study of initiation of antibody responses in cultures of rabbit spleen cells that we could obtain only marginal primary-type PFC responses. Cells in culture from rabbits pre-injected with antigen showed secondary-anamnestic-type response kinetics, but the class of antibody secreting PFC apparently remains 19S since we were unable to detect 7S, IgG secreting PFC using some of the best facilitating antisera available. This finding is contrary to published data from other laboratories and points out the importance of our discovery of an artifact in rabbit cell culture methodology described in my previous annual report.

The results of our study cited in the previous year's report relative to anti-hapten antibody combining site overlap from hapten into shared determinant regions of the hapten-carrier complex showed a small population of cells responding to this region.

Significance to Biomedical Research and the Program of the Institute: Ability to regulate and modulate specific antibody responses will have great advantange over generalized immunosuppressive methods in current use. Hopefully it will be possible to selectively suppress a response, e.g., to a grafted tissue, while maintaining desirable immunologic surveillance against disease-producing immunogens including neoplasms. Our findings should complement and help to clarify scientific thought on cellular control mechanisms in general.

Proposed Course of Project: We hope to prove the postulated activity of SPE, using the mouse model as described. Success will help to decide whether the suppressor T-cell is a separate population or merely a susceptible stage of the helper T-cell maturation. We will test further the cyclic nucleotide effect in nude cell culture in an effort to establish the potential of this small molecule as one of the soluble mediators of the helper T-cell population.

Honors and Awards:

Invited speaker at the 1974 Gordon Conference on Microbial Toxins.
Listed with the National Research Council for advisory service.

Publications:

1. Uzunova, Anelia, D., and Hanna, Edgar, E.: B-cell Amplification by N^6, O^2-dibutyryl Adenosine-3'-5' Cyclic Phosphate in Mice. Cellular Immunology 7: 507-511, 1973.
2. Hanna, Edgar, and Watson, Dennis, W.: Enhanced Immune Response Following Immunosuppression by Streptococcal Pyrogenic Exotoxin. Infection and Immunity 7: 1009-1011, 1973.

Serial No. [illegible]-6
1. Laboratory of Molecular Genetics
2.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Regulated Expression During Cell Growth and Differentiation
I. Gene expression during erythropoiesis
II. Gene expression during development of immunocompetency

Previous Serial Number: Same

Principal Investigator: P. Leder

Other Investigators:
T. Honjo
S. Orkin
S. Packman
R. Ross
D. Tiemeier
D. Swan
P. Venetianer

Cooperating Units:
[illegible]
Viral Leukemia and Lymphoma Branch, NCI

Man Years

Total:	9-3/4
Professional:	8
Other:	1-3/4

Project Description:

Objectives: To define in genetic and molecular terms the mechanisms which regulate the flow of genetic information during cell growth and development. Specific model systems involving the differentiation of red blood cell precursors and immunocytes have been chosen for detailed studies. These serve as paradigms to view the process of differentiation as well as to investigate the organization of the chromosome and its relation to the immune process.

Methods Employed: These investigations have involved a complex, inter-disciplinary approach. The model systems that have been chosen involve the maintenance in culture of a unique line of erythrocytic leukemia cells, T-3-Cl-2. This cell, when appropriately treated, undergoes a form of erythroid differentiation. Initial studies involving the immune system have focussed on several myeloma tumors which produce specific immunoglobulin light chains.

Our strategy has involved the isolation of specific mRNAs for globin and the immunoglobulin light chain using techniques of extraction and affinity chromatography developed in this laboratory. The purified mRNAs are converted into their DNA complements using the avian myeloblastosis reverse transcriptase, a procedure also developed in this laboratory. The DNA complements of these purified mRNAs are used in hybridization kinetic analyses to determine globin and immunoglobulin gene dosage and to quantitate respective mRNAs. Generally these procedures involve the use of tumors grown in animals, cloned lines grown in culture and the application of sophisticated biochemical and physical chemical techniques in the study of these systems.

Major Findings: (1) A cell-free protein synthetic system derived from wheat embryos has been established which results in a translation of immunoglobulin light chain polypeptides. (2) Using this system as an assay for immunoglobulin light chain mRNA, and several steps of purification, we have been able to purify immunoglobulin light chain message to virtual homogeneity. (3) The purified immunoglobulin message has been shown to consist of approximately 1300 nucleotides, containing approximately twice as many bases as would be required to direct the synthesis of the mature form of the immunoglobulin light chain. (4) The purified immunoglobulin mRNA has been used as a template for the synthesis of a synthetic DNA using the avian myeloblastosis reverse transcriptase. (5) This cDNA has provided a probe for hybridization studies which clearly demonstrate that the constant region of the immunoglobulin light chain is represented less than five times per haploid genome. This observation rules out a stringent germ line hypothesis which might be invoked to account for the organization of immunoglobulin genes. (6) Preliminary studies have been undertaken using iodinated immunoglobulin light chain mRNA in an effort to distinguish between the somatic mutation and germ line hypotheses which have been invoked to explain the origin of diversity of antibody molecules. (7) Studies involving the Friend leukemia virus infected erythroblastoid cell line have continued and rather rigorously quantitated the induction of globin mRNA in these cells following induction. (8) In addition, globin mRNA has been demonstrated to be incorporated into the Friend leukemia virus particles which are chronically shed by these cells in culture. (9) Several cloned lines of erythroblastoid cells have been established which can be induced to undergo erythrodifferentiation and globin mRNA accumulation in varying degrees. (10) Several of these cloned lines have been induced to form hybrids in order to study dominance relationships which govern the ability to undergo erythrodifferentiation. (11) Finally, a factor has been isolated from hematopoietic tissue which is capable of inducing erythrodifferentiation in cultured cells. This factor may represent an as yet undescribed hormone.

Significance to Biomedical Research and the Program of the Institute: These studies are directed towards understanding the fundamental processes involved in the expression of genetic information. These processes are obviously relevant to the normal and abnormal embryonic differentiation, maturation and to the orderly development and function of the human organism. In addition to the relevance of these studies to inherited disorders, the work is also relevant to the process of oncogenesis as well as to disease processes which are polygenic such as diabetes, hypertension and susceptibility to oncogenic disorders. Our current findings have provided a means for studying the regulated expression of specific genes in higher organisms. These are particularly relevant to determining the pathophysiology of thalassemia. These techniques may also prove useful in the direct transmission of genetic characteristics from cell to cell in vitro.

Proposed Course of Project: Having obtained a purified immunoglobulin mRNA, we intend to conduct careful hybridization experiments designed to distinguish between the somatic mutation and germ line hypotheses. These studies will involve the isolation of light chain mRNA from several kappa-type light chain producing myeloma tumors and their use in competition experiments with labeled immunoglobulin mRNA probe. In addition, we will begin detailed studies directed towards understanding the organization of the immunoglobulin and globin genes in the cellular genome.

Further studies will be directed towards understanding the regulated expression of globin genes in hybrid cell lines we have obtained. These hybrids represent variants with respect to their ability to undergo erythrodifferentiation. Understanding the molecular bases of these variations should provide insight into the normal regulated expression of the globin genes. Further work will be directed towards the purification and characterization of the factor we have recently identified which induces erythrodifferentiation in cultured cells. These studies will be particularly directed towards the chemical characterization of the active component.

Honors and Awards:

Invited Lectures - Symposium on Cellular Genetics, Jackson Laboratories, Bar Harbor, Maine, September 1973
Symposium on Hemoglobinopathies, New York Academy of Sciences, November 1973
Department of Biochemistry, Columbia University School of Medicine, December 1973
Philips Visitor, Department of Biology, Haverford College, Haverford, Pennsylvania, January 1974
Symposium on Immunogenetics, Squaw Valley, California, March 1974
Distinguished Lecturer, Department of Cellular Biology, Baylor Medical School, Houston, Texas, April 1974
Symposium on Genetic Organization, National Academy of Sciences, Washington, D.C., April 1974
Chairman, Gordon Conference on Nucleic Acids, New Hampton, New Hampshire, June 1974

Publications:

1. Ross, J., Aviv, H., and Leder, P.: Characterization of Synthetic Rabbit Globin DNA. Arch. Biochem. & Biophys. 158: 494-502, 1973.
2. Gielen, J., Aviv, H., and Leder, P.: Characterization of Rabbit Globin mRNA Purification by oligo(dT)-cellulose Chromatography. Arch. Biochem. & Biophys., in press.

3. Ikawa, Y., Ross, J., and Leder, P.: An Association Between Globin mRNA and 60S RNA Derived from Friend Leukemia Virus. Proc. Nat. Acad. Sci., USA, in press.
4. Leder, P., Honjo, T., Packman, S., Swan. D., Nau, M., and Norman, B.: The Organization of Immunoglobulin Genes. Proceedings of the 1974 ICN-UCLA Symp. on Molecular Biology, in press.
5. Leder, P., Ross, J., Gielen, J., Packman, S., Ikawa, Y., Aviv, H., and Swan, D.: Regulated Expression of Mammalian Genes: Globin and Immunoglobulin as Model Systems. Cold Spring Harbor Symp. on Quant. Biol., in press.
6. Ross, J., Ikawa, Y., Gielen, J., Packman, S., and Leder, P.: Induction of Globin mRNA in Friend Leukemia Virus-Infected Cells and its Presence in Viral 60S RNA. 7th International Symp. on Comparative Leukemia Research, in press.

Ninth Annual Report
July 1, 1973 through June 30, 1974
Laboratory of Biomedical Sciences

The Laboratory of Biomedical Sciences conducts basic research in developmental enzymology, intermediary metabolism, physiological controls, and developmental pharmacology.

In the Section on Developmental Enzymology interest centers on the purification and characterization of placental enzymes, of pregnancy-associated enzymes of material plasma, and of fetal enzymes. Currently under consideration are enzymes involved in the metabolism of lysine, glycogen, catecholamines, and oxytocin.

Placental lysine-α-ketoglutarate reductase and saccharopine dehydrogenase, two enzymes of lysine metabolism, have been extensively purified and characterized with respect to molecular weight, pH optimum, Michaelis constants, substrate specificity, and reaction mechanism.

The glucose-6-phosphate dependent form of glycogen synthetase has been purified extensively by taking advantage of specific complex formation between the enzyme and glycogen, which significantly changes the elution pattern of the enzyme in Agarose column chromatography. The pH optimum and Michaelis constants for the enzyme have been determined, and it has been found to be activated 5 to 7 fold by the addition of Mn^{++} and SO_4^{--} ions.

A developmental study of two catecholamine-catabolizing enzymes, catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO), in rat placenta showed that both increased significantly during the latter part of gestation. MAO, but not COMT, underwent a significant decrease between the 20th day and the time of parturition. The mean COMT activity in human term placenta was about one-half of that in rat term placenta; the mean MAO activity of the human placenta was about five times that of the rat placenta. Very little COMT activity, and no MAO activity, was found in choriocarcinoma cells grown in culture.

Specific activities and zymograms of cystine aminopeptidase (CAP) and leucine aminopeptidase (LAP) were compared among human placenta, rhesus monkey placenta, and choriocarcinoma grown in culture. The CAP band of choriocarcinoma, was found to have the same electrophoretic mobility as CAP_1 of human pregnancy serum.

Investigators in the Section on Intermediary Metabolism develop laboratory analogs of mental retardation through the use of specific chemical agents and enzyme inhibitors, investigate the biosynthesis and degradation of macromolecules in neural tissue, and elucidate mechanisms of transport of small molecules into and out of the brain. The studies in the Section range from classical biochemical investigations of enzymes of neurochemical interest and pharmacological efforts to inhibit or stimulate these enzymes to behavioral experiments on animals in which the activity of such enzymes has been altered or inhibited. In this way we hope to provide information on the basic biochemistry of the brain as well as on the conditions which alter the normal brain functions such as various genetically-transmitted mental retardation syndromes.

During the past year the purification to homogeneity of GTP-cyclohydrolase, the first enzyme in the pteridine biosynthetic pathway, and the identification of its product as D-erythro-neopterin triphosphate was accomplished.

The further purification and characterization of bacterial tryptophan hydroxylase and bacterial dihydropteridine reductase was carried out.

A third enzyme, also involved in bacterial hydroxylation, phenylalanine hydroxylase, has been studied and its mechanism of control by product and product metabolites has been elucidated.

Procedures for RNA extraction from brain which minimize messenger RNA destruction were developed, permitting the demonstration that all messenger RNA activity in brain is associated with RNA's having poly A sequences.

Rats treated with high phenylalanine and p-chlorophenylalanine during discrete periods of brain development were characterized by permanent histologic changes (focal areas of abnormal myelination and progressive gliosis) in optic nerve, by delayed eye-opening and cataract formation when treated prior to 10 days of age, and by a high fetal blood and brain level of phenylalanine.

The Section on Physiological Controls studies basic endocrine and neuroendocrine mechanisms and factors controlling their development. A focus on the synthesis and the mode of action of cyclic AMP reflects the potential importance of this ubiquitous intracellular regulator to our understanding of metabolic regulation, cell growth, and differentiation. Two metabolic pathways are used for delineating interactions between hormones, neurotransmitters, and nutrients. Protein phosphorylations controlling glycogen metabolism provides us with a well-characterized model of cyclic AMP action; indole metabolism in the pineal gland has proved excellent for studying the mode of action and synthesis of neurotransmitters.

Additional regulation of glycogen cycle enzymes has been delineated. We have further purified a metal-activated phosphoprotein phosphatase which appears to reverse specifically the effects of the cyclic AMP-dependent protein kinase. It has been separated from phosphorylase phosphatase. Phosphorylase phosphatase has been found to be a third enzyme in glycogen metabolism which is controlled (inactivated) by the cyclic AMP-dependent kinase.

Studies on regulation of fetal rat liver glycogen synthesis have been facilitated by the development of methodology for culturing fetal hepatocytes free of fibroblasts. Adrenal glucocorticoids specifically initiate the developmental appearance of glycogen synthetase in this system.

Investigations on adrenergic (cyclic AMP) stimulation of pineal gland serotonin N-acetyltransferase indicated a requirement for membrane hyperpolarization. Increased enzyme activity caused by norepinephrine is blocked by agents which block membrane hyperpolarization. These agents (ouabain and high concentration of extracellular potassium) do not block the increased cyclic AMP levels that occur following norepinephrine. These findings led to the hypothesis that cyclic AMP acts only in a specific intracellular ionic environment which is established by norepinephrine.

The Section on Developmental Pharmacology studies the subcellular processes involved in the "induction" of pharmacologically important enzymes by drugs, polycyclic hydrocarbons, or insecticides (i.e., xenobiotics). The combination of these environmental stimuli and the host's genetic constitution influence the levels of these monooxygenase activities in maternal tissues, in the placenta, and transplacentally in fetal tissues. Consequently, we are concerned with (1) the interrelationship between genetic expression and environmental stimuli, (2) possible effects of xenobiotics on normal fetal growth and development, (3) mechanisms of "pharmacological immaturity" of the premature and newborn, (4) mechanisms of disease of the fetus and newborn involving oxidative and conjugative metabolism of normal body substrates such as steroids and bilirubin, and (5) possible therapeutic and diagnostic procedures for aiding and predicting pharmacogenetic disorders and teratogenic effects in the fetus and neonate.

With the use of inbred strains of mice, we have determined that the "induction" of at least eight drug-metabolizing enzyme activities can segregate as a single autosomal dominant or codominant trait or the lack of "induction" can be expressed dominantly--depending on the inbred strains crossed. The simplest genetic model which fits most of the data is regulated by two non-linked loci, each having at least three alleles, or gene products; the possibility of an interstrain-specific suppressor gene is also being investigated. A new hemoprotein, cytochrome P$_1$450, is always concomitantly synthesized during the induction process. In mice in which the induction process and P$_1$450 formation are relatively "nonresponsive" to aromatic hydrocarbons such as 3-methylcholanthrene or β,6-benzoflavone, these parameters are fully expressed by administration of a compound having extremely high avidity for all tissues, 2,3,7,8-tetrachlorodibenzo-p-dioxin; it thus appears that the "nonresponsive" mice have the necessary regulatory and structural genes necessary for cytochrome P$_1$450 formation and induction of these enzyme activities and that the difference reflects a defective "receptor binding-site" for the aromatic hydrocarbon inducers in the "nonresponsive" mice.

By means of reconstitution experiments with three submicrosomal fractions, we have shown that the genetic difference in inducible drug-metabolizing enzyme activity resides entirely in the CO-binding cytochrome fraction.

With fetal cell cultures derived from the entire hamster or mouse, from rat or mouse liver, and most recently from established cell lines of rat or mouse hepatoma or normal liver origin, we have developed an experimental model having certain advantages over studies in the intact animal. However, of the two possible forms of aryl hydrocarbon hydroxylase activity found in the intact animal, we have discovered that only one form is induced in fetal liver or hepatoma cell culture, regardless of the type of inducer used in the growth medium; we suggest that one should therefore be cautious in the extrapolation of certain data from cell culture to results found in the intact animal. Other recent developments include (1) the demonstration that genetic differences in diphenylhydantoin hydroxylation exist between inbred mouse strains; (2) the finding that genetic differences in carcinogenesis by 3-methylcholanthrene but not benzo[a]pyrene or 7,12-dimethylbenz[a]anthracene *in vivo* parallel the differences we find with mutagenesis by these chemicals *in vitro*, using the histidine-dependent bacterial tester strains of Bruce Ames; and (3) a method for distinguishing between the homozygote and heterozygote "responsive" mouse. The relative presence of "aromatic hydrocarbon responsiveness" in certain mice results in more 3-methylcholanthrene-initiated tumors, in a shortened sleeping time when given certain drugs, in a shortened survival time when dosed with large amounts of polycyclic hydrocarbons, and in an increased protection against the toxicity of certain insecticides. These genetically mediated dissimilarities in xenobiotic metabolism may be a useful probe in demonstrating differences in the rates of formation of certain drug-produced birth defects.

Serial No. HD-LB-1
1. Laboratory of Biomedical Sciences
2. Section On Developmental Enzymology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Developmental Enzymology

Previous Serial Number: HD-LB1(c)

Principal Investigator: J. C. Robinson

Other Investigators: C. Chen
K. P. Huang
T. Fjellstedt
T. Ota

Cooperating Units: None

Man Years

Total: 48/12
Professional: 48/12
Other: 0/12

Project Description:

Objectives: The main objectives of this project are: (1) to study qualitative and quantitative changes in selected enzyme systems in the developing placenta and fetal organs in normal gestation; (2) to study qualitative and quantitative changes in these same enzyme systems in the developing placenta and fetal organs in reproductive disorders such as abortions, stillbirths, and fetal malformations; (3) to isolate, purify, and characterize enzymes from the normal placenta; and (4) to identify and characterize pregnancy-associated enzymes in maternal blood plasma.

Methods Employed: The principal methods employed are: (1) protein isolation technques, including salt- and solvent-fractionation, gel-filtration, and ion-exchange chromatography; (2) enzyme assays, which are usually based on spectrophotometric or radiometric methods; (3) the zymogram technique, with which electrophoretic variants of enzymes can be detected; (4) gel-autoradiography, with which potential carrier function of a particular protein is evaluated; (5) paper and thin layer chromatography; (6) tissue culture; and (7) immunochemical procedures.

Major Findings: (1) Column chromatographic procedures resulted in an approximately 2,000 fold purification of the lysine metabolizing enzymes, lysine-α-ketoglutarate reductase and saccharopine dehydrogenase, and product inhibition experiments were used to define the reaction mechanism of the conversion of lysine to saccharopine. The demonstrated inability of this enzyme to mediate the conversion of saccharopine to lysine was attributed to the extremely high binding constant found for the NADP cofactor. The molecular weight of each enzyme was found to be approximately 480,000, and the inability to separate the two activities by ion exchange chromatography, affinity chromatography, gel filtration, and ultracentrifugation--the latter two carried out both in the presence and in the absence of detergent--led to the assumption that the two activities are either bound together directly or through a membrane fragment. (2) The glucose-6-phosphate dependent form of glycogen synthetase of human placenta has been purified over 4,000 fold by taking advantage of specific complex formation between the enzyme and glycogen, which significantly changes the elution pattern of the enzyme in Agarose column chromatography. The pH optimum of the reaction was established. In the absence of glucose-6-phosphate, the enzyme was activated two to three fold by the addition of either 5 mM $MnCl_2$ or 10 mM $(NH_4)_2SO_4$; addition of both 5 mM $MnCl_2$ and 10 mM $(NH_4)_2SO_4$ caused a five to seven fold activation. (3) A developmental study of the catecholamine metabolizing enzymes, catechol-O-methyl transferase (COMT) and monoamine oxidase (MAO), in rat placenta showed that both increased significantly during the latter part of gestation. Between day 20 and day 22 (at the time of parturition) a significant decrease in MAO activity was observed; during this same period COMT activity remained essentially unchanged. The mean COMT activity in human term placenta was about one-half of that in rat term placenta; the mean MAO activity of the human rat placenta was about five times that of the rat placenta. Very little COMT activity, and no MAO activity, was found in choriocarcinoma cells grown in culture. (3) The specific activity of cystine aminopeptidase (CAP) in choriocarcinoma cells from culture was found to be 1.5 fold greater than the specific activity of term placenta. The extract of choriocarcinoma contained CAP which is similar to CAP_1 of human pregnancy serum in electrophoretic mobility and in resistance to inhibition by 0.1 M methionine. Growing the cells in a wide range of concentrations (10^{-5} to 10^{-1} units/ml) of oxytocin did not increase the specific activity of either the cells or of the medium. The specific activities and zymograms of CAP and leucine aminopeptidase (LAP) were compared among human placenta, rhesus monkey placenta, and choriocarcinoma grown in culture.

Significance to Biomedical Research and the Program of the Institute: The study of placental enzymes in normal pregnancy will provide a suitable baseline with which to compare the same enzyme systems in placental explants, in benign and malignant trophoblast cells in culture, and in placental tissue from selected reproductive disorders. The elucidation of the lysine metabolic pathway is fundamental to investigation of hyperlysinemia and saccharopinuria, two genetic diseases resulting in mental retardation and motor abnormalities. The study of the regulation of glycogen synthetase in the human placenta is essential for the understanding of fetal carbohydrate metabolism which is greatly influenced by the placenta before the fetal liver has fully developed. The investigation of COMT is important because steroids, as well as catecholamines, are substrates of this methyltranferase. The study of CAP was undertaken because this enzyme, in maternal serum, is being used for the assessment of placental function. Yet the origin of CAP in pregnancy serum has not been unequivocally established. This research has shed some light on this problem. Hopefully, these studies will lead to increased understanding of both normal and abnormal biochemical processes involved in pregnancy and fetal development.

Proposed Course of Project: (1) Characterization of selected enzymes from placenta, maternal plasma, amniotic fluid, and trophoblast cells in culture will continue. (2) Correlations between abnormal pregnancies and variations in fetal or maternal enzymes will be sought.

Honors and Awards: None

Publications:

1. Chen C. H., Klein, D. C., and Robinson, J. C.: Catechol-O-methyltransferase in rat placenta, human placenta, and choriocarcinoma grown in culture. J. Reprod. Fertil., in press.

Serial No. HD-LB-9
1. Laboratory of Biomedical Sciences
2. Section on Intermediary Metabolism
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Effect of Metabolic Disorders on the Biochemistry, Structure, and Function of the Developing Brain

Previous Title: Models of Mental Retardation

Previous Serial Number: Same

Principal Investigator: G. Guroff

Other Investigators:
A. Andersen
V. Rowe
L. Avins

Cooperating Units:
T. Kuwabara, NEI, NIH, Bethesda, Md.
Henry Fales, NHLI, NIH, Bethesda, Md.
John Pisano, NHLI, NIH, Bethesda, Md.

Man Years

Total:	34/12
Professional:	34/12
Other:	0/12

Objectives: The objectives of this research are: (1) to study in detail changes in brain chemistry and morphology in the developing rat resulting from a specific metabolic abnormality, hyperphenylalaninemia; (2) to attempt to reverse abnormal brain function produced by such treatment by using physiological methods; (3) to study the development of physiological systems for regulating entry of metabolites into the brain.

Methods Employed: Standard assay techniques for amino acids, serotonin, and enzymes; electron microscopic and light microscopic examination; gas chromatography and mass spectroscopy; behavioral evaluation.

Major Findings: (1) The large neutral amino acids (leucine, isoleucine, valine, tryptophan and tyrosine) compete with phenylalanine for entry into the brain of the neonatal rat pup. When these amino acids are given along with phenylalanine, the brain phenylalanine level resulting is lower than when phenylalanine is given alone. (2) The fetal rat experiences a phenylalanine blood and brain level which is several times that experienced by the maternal rat. (3) The fetal rat is less well able to protect the brain against high blood phenylalanine levels than the adult rat. (4) Rats treated with a combination of p-chlorophenylalanine plus phenylalanine during the period 10-30 days of age do not show delayed eye opening or cataract formation, suggesting that vulnerability in regard to these two measures of development occur during the first 10 days of development. (5) When adult rats treated in the pre-weaning state with a combination of p-chlorophenylalanine plus phenylalanine are compared with adult rats given control saline injections in the pre-weaning period, there is no difference between these groups in brain serotonin, tyrosine hydroxylase, or tryptophan hydroxylase. (6) The major urinary metabolites of low doses of phenylalanine given to pre-weaning rats are: phenaceturic acid and hippuric acid. In addition, phenyllactic acid and various phenolic acids are excreted in small quantities. At higher doses of phenylalanine, the typical unconjugated metabolites appear in the urine, including phenyllactic acid, phenylpyruric acid, and mandelic acid. (7) Early treatment with p-chlorophenylalanine plus phenylalanine produces permanent histologic changes in the optic nerve of rat, especially abnormal myelination and gliosis.

Significance to Biomedical Research and the Program of the Institute: The current treatment of phenylketonuria has been to limit phenylalanine in the diet. Disadvantages of this method include difficulty in preparing food with artificial low phenylalanine mixtures, unpalatability of the diet, and growth retardation if phenylalanine is too strictly limited. It may be possible to limit phenylalanine in the brain by using basic concepts of competition for transport systems into the brain, supplementing the diet with large neutral amino acids (other than phenylalanine) instead of limiting phenylalanine in the diet. The lack of an adequate blood-brain barrier against phenylalanine in the fetal rat brain, plus the presence of a phenylalanine level in the fetus which is several times that of the maternal rat, helps to explain why PKU women with phenylalanine levels as low as 14 mg% frequently have malformed or non-viable children. The studies on the metabolism of phenylalanine in the rat are important because many extrapolations of data are made from experimental animals to the clinical situation, yet the validity of this extrapolation is often not examined. The electron microscopic work demonstrates and documents the time course of the permanent neurological damage resulting from neonatal hyperphenylalaninemia.

Proposed Course of Project: (1) Detailed studies of the effect of high phenylalanine levels in the fetal and neonatal rat on the level of other amino acids in the brain, on protein synthesis, and on gross brain development. (2) Attempts will be made to reverse the abnormal changes produced by hyperphenylalanemia by supplementing the diet with those amino acids lowered by high phenylalanine. (3) Experiments will be designed to determine whether animals treated with a regimen previously shown to produce permanent behavioral and neuropathological damage, but supplemented with amino acids competing with phenylalanine for entry into the brain, can produce normal brain development. (4) A study of the development of the blood brain barrier with regard to the transport system for large neutral amino acids. (5) Experiments designed to test for hyperactivity and other behavioral changes in animals treated during the 10-30 day period with p-chlorophenylalanine plus phenylalanine.

Honors and Awards: None

Publications:

1. Andersen, A.E., Abramowitz, A.Z., and Guroff, G: Biochemical and behavioral changes in rats with experimental phenylketonuria. In Barchas, J., and Usdin, E. (Eds.): Serotonin and Behavior. New York, Academic Press, 1973, pp. 335-349.
2. Rowe, V.D., Zigler, S., Andersen, A.E., Sidbury, J.B., and Guroff, G.: Some characteristics of the p-chlorophenylalanine-induced cataract. Exp. Eye Res. 17: 245-250, 1973.
3. Andersen, A.E., Rowe, V., and Guroff, G.: The enduring behavioral changes in rats with experimental phenylketonuria. Proc. Nat. Acad. Sci. 71: 27-25, 1974.
4. Andersen, A., and Guroff G.: Phenylketonuria in rats. Comp. Path. Bulletin 5: 2-4, 1974.

Serial No. HD-LB-10
1. Laboratory of Biomedical Sciences
2. Section on Intermediary Metabolism
3. Bethesda

Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mechanism of Aromatic Hydroxylation

Previous Serial Number: Same

Principal Investigator: G. Guroff

Other Investigators:
C. Letendre
C. Williams

G. Dickens

Cooperating Units: T. Shiota, Department of Microbiology, University of Alabama, Birmingham, Alabama

Man Years:

Total:	38/12
Professional:	26/12
Others:	12/12

Project Description:

Objectives: The objectives of this research are: (1) to provide information on the process of enzymatic hydroxylation and (2) to get detailed information on the nature of phenylalanine hydroxylase.

Methods Employed: The methods employed include standard techniques of enzyme kinetics and purification, antibody detection, and organic synthesis and characterization.

Major Findings: (1) Chromobacter tryptophan hydroxylase, which had not previously been described in bacteria, has been purified and characterized, and its sulfhydryl requirement and pteridine specificity determined. (2) Dihydropteridine reductase, an enzyme which is instrumental in providing a source of reduced pteridines in Pseudomonas, has been further purified and characterized. (3) The study of phenylalanine hydroxylase from Pseudomonas has been continued with particular reference to the mechanism of metal activation and to the control and regulation of enzyme activity by way of the metal activation step.

Significance to Biomedical Research and the Program of the Institute: Information about the phenylalanine and tryptophan hydroxylases and about ways to inhibit or stimulate them relates directly to the general problems of normal neural function and of mental retardation. Phenylalanine hydroxylase is the missing enzyme in the best characterized of the genetic retardations, phenylketonuria. Tryptophan hydroxylase is the first and rate-limiting enzyme in the biosynthesis of the neurohumoral agent, serotonin. Detailed analysis of the structure and mechanism of these enzymes will provide new and unique information since relatively little has been accomplished on hydroxylases in general and few, if any, pteridine enzymes have been reported as homogenous preparations.

Proposed Course of Project: (1) Continued study of the purification, mode of action, and structure of the bacterial enzymes tryptophan hydroxylase, phenylalanine hydroxylase, and dihydropteridine reductase. The interrelationship of the latter two enzymes and the mechanism of action and the role of a metal ion will be studied in more detail. (2) A study of phenylalanine hydroxylase in mammalian organ and tissue cultures will be undertaken with a goal of elucidating the time course of appearance of enzyme activity and the effect of hormones, phosphatases, and kinases in the appearance of enzyme activity in fetal tissues.

Honors and Awards: None

Publications: None

Serial No. HD-LB-11
1. Laboratory of Biomedical Sciences
2. Section on Intermediary Metabolism
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biosynthesis and Function of Pteridines

Previous Serial Number: Same

Principal Investigator: G. Guroff

Other Investigators: J. Cone
J. Plowman

Cooperating Units: None

Man Years:

Total:	14/12
Professional:	7/12
Other:	7/12

Project Description:

Objectives: The objectives of this research are: (1) to provide information on the enzymes in the biosynthetic pathway to the pteridine ring and (2) to investigate the functions of pteridines in addition to their action as hydroxylase cofactors.

Methods Employed: The methods used include standard techniques of enzyme purification, isotope measurement, high-voltage electrophoresis, and fluorescence detection.

Major Findings: (1) The enzyme, GTP-cyclohydrolase, from *Pseudomonas* has been purified to homogeneity by use of an affinity column, and a stimulating factor has been isolated and characterized; (2) the isolation and identification with respect to state of oxidation, degree of phosphorylation, and steric configuration of the side chain of the first product of pteridine biosynthesis has been accomplished. This product is D-*erythro*-dihydroneopterin triphosphate.

Significance to Biomedical Research and the Program of the Institute: The pteridine cofactors are important in a number of hydroxylase reactions including those leading to the neurohormones serotonin and norepinephrine. Information about the pharmacology and biochemistry of these biosynthetic enzymes may lead to the ability to inhibit or stimulate these pathways and perhaps to new therapeutic tools for maladies involving these neurohormones.

Proposed Course of Project: (1) Purification and characterization of other enzymes of pteridine metabolism; (2) attempts to link pteridines or pteridine derivatives to the enzymes they serve; (3) study of pteridine biosynthetic enzymes in photosynthetic organisms; (4) search for other functions of pteridines.

Honors and Awards: None

Publications:

1. Cone, J., Plowman, J., and Guroff, G.: Purification and properties of GTP-cyclohydrolase and its stimulating protein from *Comamonas sp.* *J. Biol. Chem.*, in press.

2. Plowman, J., Cone, J., and Guroff, G.: Identification of D-*erythro*-dihydroneopterin triphosphate, the first product of pteridine biosynthesis in *Comamonas sp.* *J. Biol. Chem.*, in press.

Serial No. HD-LB-12
1. Laboratory of Biomedical Sciences
2. Section on Intermediary Metabolism
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biochemistry of Brain Macromolecules

Previous Serial Number: Same

Principal Investigator: G. Guroff

Other Investigators:
J. DeLarco
S. Nakagawa
K. Bromwell

Cooperating Units: None

Man Years:

Total: 27/12
Professional: 15/12
Others: 12/12

Project Description:

Objectives: The objectives of this research are: (1) to investigate the synthesis and function of brain macromolecules; and (2) to compare, characterize, and identify the products made in a cell-free protein synthesizing system using different populations of messenger RNA's obtained from brain organelles.

Methods Employed: The methods employed in these studies include those used to isolate and characterize messenger RNA's (i.e. extraction, centrifugation, hybridization to oligo(dT)-cellulose, and gel electrophoresis), cell-free protein synthesis using radioactive amino acid, and gel electrophoresis in order to characterize the products from the cell-free protein synthesis.

Major Findings: (1) The messenger RNA's associated with brain polysomes are more susceptible to nuclease destruction than the messenger RNA's associated with the polysomes from liver; (2) methods for extracting RNA's have been worked out which minimize the destruction of the mRNA's; (3) a cell-free protein synthesizing system which utilizes brain messenger RNA to direct the incorporation of amino acids into an acid-precipitable fraction has been found; (4) all of the messenger RNA activity in brain is associated with the RNA's having poly(A) sequences; (5) polysomes, synaptosomes, and mitochondira isolated from rat brain have poly(A)-containing RNA's which efficiently direct the incorporation of amino acids into an acid-precipitable fraction in the wheat germ cell-free protein synthesizing system.

Significance to Biomedical Research and the Program of the Institute: The study of macromolecules in brain organelles such as synaptosomes can give us a means of increasing our understanding of normal neuronal transmission. An investigation of synaptosomal messenger RNA's has therefore been initiated, since these are the macromolecules central to nucleic acid and protein metabolism. The understanding of the synthesis and function of the RNA's within these systems may provide tools for the investigation of the biochemical alteration occurring in neural disorders.

Proposed Course of Project: Further examination of synaptosomal RNA's in order to determine: (1) the location of these RNA's within the synaptosomes; that is, are these RNA's contained within the synaptosomal mitochondria or are they also located on the synaptic membranes and in the synaptosol; (2) whether these RNA's originate in the nucleus and are transported to the synaptosomes by axoplasmic flow or are synthesized locally (i.e. within the mitochondria); (3) the function of these RNA's; that is, do these RNA's code for the synthesis of specific proteins required for synaptic function.

Honors and Awards: None

Publications:

1. Nakagawa, S., and Guroff, G.: The uptake of purine by rat brain *in vivo* and *in vitro*. J. Neurochem. 20: 1143-1149, 1973.

2. DeLarco, J., Abramowitz, A., Bromwell, K., and Guroff, G.: Polyadenylic acid-containing RNA from rat brain. *J. Neurochem.*, in press.

Serial No. HD-LB-4
1. Laboratory of Biomedical Sciences
2. Section on Physiological Controls
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Development of Mammalian Metabolic Regulation

Previous Serial Number: Same

Principal Investigator: W. H. Glinsmann

Other Investigators:
H. Eisen
F. Zieve
F. Huang

Cooperating Units:
R. Chez, Pregnancy Research Branch, NICHD
K. Freude, Minneapolis Veterans Hospital, University of Minnesota
L. Zieve, Minneapolis Veterans Hospital, University of Minnesota
P. Sherline, Department of Medicine, Washington University School of Medicine, St. Louis, Mo.

Man Years

Total:	63/12
Professional:	50/12
Others:	13/12

Project Description:

Objectives: This project seeks to define in mammalian cells: (1) mechanisms underlying differential modulation of carbohydrate and lipid metabolism and adaptive protein synthesis by hormones and nutrients, and (2) factors controlling development of these mechanisms. A general model is being examined based on control of protein phosphorylations which regulate rate-limiting enzymes. Current interest centers on a comprehensive description of glycogen cycle enzymes.

Methods Employed: Isolated cell and organ culture techniques and isolated organ perfusion are used in studying liver glucose production and rate-limiting enzymes in glycogen metabolism. Enzymes and regulatory proteins controlling glycogen turnover are isolated; their mode of regulation is described.

Major Findings: (1) Phosphoproteins controlling glycogen turnover: Many details of how cyclic AMP mediates the effects of glucagon and epinephrine on glycogenolysis are known: cyclic AMP-activation of a general protein kinase which phosphorylates regulatory proteins has been studied extensively. However, increasing evidence supports the concept that hormonal and substrate effects on protein phosphorylation are also mediated through the corresponding phosphatase reactions. We are developing a detailed model for studying these interactions based on separation of glycogen cycle enzymes.

We postulated the existence of a general phosphoprotein phosphatase that reverses the effects of the cyclic AMP-dependent protein kinase. This general phosphatase has now been characterized as a metal-activated enzyme which dephosphorylates glycogen synthetase, phosphorylase kinase, phosphohistone, and protamine. It has been separated from metal-independent histone phosphatases and phosphorylase phosphatase.

Cyclic AMP-dependent protein kinase is known to control the activity of two enzymes of glycogen metabolism, glycogen synthetase and phosphorylase kinase. We have now obtained evidence that the activity of a third glycogen cycle enzyme, phosphorylase phosphatase, is also specifically controlled (inactivated) by protein kinase. In studying activation and inactivation reactions of phosphorylase phosphatase, evidence was obtained that a second cyclic AMP-independent kinase is involved.

(2) Development of hormonal and substrate regulation of glycogen turnover in isolated fetal liver: We have previously shown with explants of fetal rat liver that hydrocortisone stimulates the initial induction of glycogen synthetase and that insulin activates this enzyme and promotes glycogen synthesis. Explants are limited for further studies in hormonal regulation because of restricted diffusion of macromolecules. We have now developed methodology for maintaining fibroblast-free primary cultures of fetal rat liver hepatocytes which are not diffusion-limited. Adrenal glucocorticoids have been shown to induce specifically glycogen synthetase in these cells.

Comparisons are being made on the development of hepatic glucose regulation between the rat and monkey. Isolated perfused term fetal Rhesus monkey livers have responses to glucagon and to altered glucose concentration which are very similar to those observed with isolated term fetal rat liver. They rapidly mobilize glycogen and release glucose in response to glucagon and hypoglycemia but are unable rapidly to store glucose as glycogen during hyperglycemia.

(3) Phosphoproteins controlling lipid metabolism: Both lipogenesis and lipolysis are under hormonal influence. Regulation of lipolysis involves the interconversion of two forms of hormone-sensitive lipase mediated by cyclic AMP-dependent protein kinase. In continuing our search for other enzymatic substrates of the cyclic AMP-dependent protein kinase, we have investigated two rate-limiting enzymes involved in lipogenesis. Our present evidence indicates that neither acetyl CoA-carboxylase nor monoglyceride esterase is directly phosphorylated by the cyclic AMP-dependent kinase.

Significance to Biomedical Research and the Program of the Institute: Regulation of cellular metabolism, growth, and differentiation at many phylogenetic levels is in part mediated by a complex series of interactions between glucose and factors which alter cyclic AMP metabolism. Currently, the best characterized model for cyclic AMP action in mammalian tissue (where cyclic AMP acts as an intracellular mediator for many hormones and neurotransmitters) is the cascade of glycogen cycle protein phosphorylations initiated by cyclic AMP. We and others have shown that these reactions are also regulated by glucose, insulin, and glucocorticoids. Therefore a comprehensive decription of these reactions will hopefully allow us to develop a general model for cyclic AMP-glucose interactions in mammalian cells and thus provide a basis for detailed studies on factors affecting the development of the corresponding regulatory systems. The control of lipid metabolism is another system in which protein phosphorylations play an important regulatory role and in which interactions among hormones, substrates, and cyclic AMP can be studied.

Proposed Course of Project: Separation and characterization of enzymes participating in glycogen cycle phosphorylations will continue with particular emphasis on the regulatory roles of a general phosphoprotein phosphatase and phosphorylase phosphatase. Possible mechanisms mediating the effects of glucose and insulin will be sought.

Primary fetal rat hepatocyte cultures will be used to further define factors influencing the development of rate-limiting enzymes or hormone receptors that control glucose homeostasis, glycogen metabolism, gluconeogenesis, and cyclic AMP turnover.

Honors and Awards: None

Publications:

1. Eisen, H.J., Goldfine, I.D., and Glinsmann, W.H.: Regulation of hepatic glycogen synthesis during fetal development: Roles of hydrocortisone, insulin, and insulin receptor. Proc. Nat. Acad. Sci. (USA) 70: 3454-3457, 1973.

2. Sherline, P., Eisen, H.J., and Glinsmann, W.H.: Acute hormonal regulation of cyclic AMP content and glycogen phosphorylase activity in fetal liver in organ culture. Endocrinology 94: 935-939, 1974.

Serial No. HD-LB-18
1. Laboratory of Biomedical Sciences
2. Section on Physiological Controls
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Regulation of Neuroendocrine Metabolism

Previous Serial Number: Same

Principal Investigator: D. C. Klein

Other Investigators: A. Parfitt

Cooperating Units:
R. Y. Moore, Division of Neurology, University of Chicago Medical School, Chicago, Illinois
A. Yuwiler and A. Geller, Neurobiochemistry Laboratory, V.A. Brentwood Hospital, Los Angeles, California
B. Marks and K. Sakai, Department of Pharmacology, Ohio State University Medical School, Columbus, Ohio
M. Vaughan, Department of Anatomy, University of Texas at San Antonio, San Antonio, Texas

Man Years

Total:	36/12
Professional:	24/12
Others:	12/12

Project Description:

Objectives: (1) To describe the molecular mechanism through which neuronal activity regulates biochemical activity in a neuroendocrine tissue, the pineal gland, and in the brain. (2) To describe the effects of arginine vasotocin and other pineal peptides on reproductive function.

Methods Employed: (1) Organ culture is used to provide a well defined and easily controlled experimental system for studying neurochemical regulation. Tissues are cultured and treated with drugs and relevant neurochemicals. The effects of these treatments are determined by measuring: (a) the amount of cyclic AMP in the tissue; (b) the activity of the enzyme that degrades cyclic AMP; (c) the activity of N-acetyltransferase, the enzyme that converts serotonin to N-acetylserotonin; (d) the amount of serotonin in the gland and culture medium; and (f) the conversion of tryptophan to serotonin and serotonin derivatives. (2) Arginine vasotocin and other pineal peptides are injected into experimental animals, and the weights of reproductive organs and other parameters of reproductive function are determined.

Major Findings: (1) Neural regulation of biochemical activity: The hyperpolarizing effect of norepinephrine is markedly reduced by ouabain or a high concentration of potassium. These agents do not block the norepinephrine-caused increase in cyclic AMP, but do block the increase in the activity of N-acetyltransferase. In addition, ouabain or a high concentration of potassium blocks the increase in enzyme activity caused by dibutyryl cyclic AMP. (2) It was found that arginine vasotocin and purified pineal peptides inhibit the growth of reproductive organs in rats and mice. It was also found that the effects of a pineal peptide and arginine vasotocin were blocked by treatment with sulfhydryl reagents. In the case of arginine vasotocin, which in an unreduced state is antigonadotrophic, the reduced form was gonadotrophic.

Significance to Biomedical Research and the Program of the Institute: These studies have provided insight into the details of one mechanism which serves to regulate indole metabolism in the pineal gland. Indole metabolism appears to be involved in regulating growth and function of reproductive organs, presumably via the hypothalamus. It is possible that indole compounds themselves are active antigonadotrophic compounds. Alternatively, indole metabolism in the pineal gland might function to regulate the production and release of pineal peptides which are involved in the antigonadotrophic effects of the pineal gland.

Alterations in this regulatory mechanism might be responsible for delayed or accelerated puberty and other abnormalities which arise during development of the reproductive system. In addition, the pineal gland may also be involved in changes in reproductive function seen in aging.

Proposed Course of Project: (1) The regulation of neurochemical metabolism will have as its focus the regulation of the activity of N-acetyltransferase. The nature of the interaction between cyclic AMP and ion physiology will be explored. (2) Investigations into the nature and effects of pineal peptides and the mechanism regulating the synthesis and release of pineal peptides will be continued. (3) An examination of the regulation of indole metabolism in the brain will be initiated.

Honors and Awards: Appointment of the Principal Investigator to the
Editorial Board of Neuroendocrinology.

Publications:

1. Klein, D.C.: The role of serotonin N-acetyltransferase in the adrenergic regulation of indole metabolism in the pineal gland. In Barckas, J. and Usdin E. (Eds.): Serotonin and Behavior. New York, Academic Press, 1973, pp. 109-120.

2. Klein, D.C.: Circadian rhythms in indole metabolism in the rat pineal gland. In Schmitt, F. O., and Worden, F. G. (Eds.): The Neurosciences: Third Study Program. Cambridge, MIT Press, 1973, pp. 509-516.

3. Klein, D.C. Yuwiler, A., Weller, J.L., and Plotkin, S.: Postsynaptic adrenergic-cyclic AMP control of the serotonin content of cultured rat pineal glands. J. Neurochem. 21: 1261-1272, 1973.

4. Binkely, S.A., MacBride, S., Klein, D.C., and Ralph, C.: Pineal enzymes: Regulation of avian melatonin synthesis. Science 181: 273-275, 1973.

5. Coyle, J.T., Jacobowitz, D., Klein, D.C., and Axelrod, J.: Dopaminergic neurons in explants of substantia nigra in culture. Neurobiology 4: 461-470, 1973.

6. Klein, D.C. and Yuwiler, A.: Beta-adrenergic regulation of indole metabolism in the pineal gland. In Usdin, E. and Snyder, S. (Eds.): Frontiers in Catecholamines. New York, Pergamon Press, 1973, pp. 321-325.

7. Binkley, S., Klein, D.C., and Weller, J.: Dark induced increase in pineal serotonin N-acetytransferase activity: A refractory period. Experimentia 29: 1339-1340, 1974.

8. Ulrich, R., Yuwiler, A., Wetterberg, L., and Klein, D.C.: Effects of light and temperature on the pineal gland in suckling rats. J. Neuroendocrinology 13: 201-320, 1974.

9. Moore, R.Y. and Klein, D.C.: Visual pathways and the central neural control of a circadian rhythm in pineal serotonin N-acetyltransferase activity. Brain Research 70: 17-33, 1974.

10. Bensinger, R.E., Klein, D.C., Weller, J.L., and Lovenberg, W.M.: Radiometric assay of total tryptophan hydroxylation by intact cultured pineal glands. J. Neurochem., in press.

11. Chen, C., Klein, D. C., and Robinson, J. C.: Catechol-O-methyltransferase in human placenta and choriocarcinoma grown in culture. Journal of Reproduction and Fertility, in press.

12. Vaughan, M.K., Vaughan, G.M., and Klein, D.C.: Arginine vasotocin: Effects on the development of reproductive organs. Science, in press.

Serial No. HD-LB-8
1. Laboratory of Biomedical Sciences
2. Section on Developmental Pharmacology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Developmental Pharmacology

Previous Serial Number: HD-LB-8(1)

Principal Investigator: D. W. Nebert

Other Investigators:
I. S. Owens
J. R. Robinson
A. Niwa
J. S. Felton
N. Considine
K. Kumaki
S. A. Atlas
S. S. Thorgeirsson

Cooperating Units:
E. B. Thompson, Laboratory of Biochemistry, NCI
J. W. Daly, Laboratory of Chemistry, NIAMD
H. Kon, Laboratory of Chemical Physics, NIAMD
M. J. Coon, University of Michigan, Ann Arbor
A. P. Poland, University of Rochester Med. Sch., N.Y.
R. E. Kouri, Microbiological Associates, Inc., Bethesda

Man Years

Total	97/12
Professional	85/12
Others:	12/12

Project Description:

Objectives: Membrane-bound mixed-function oxygenases are important in the metabolism of drugs, carcinogens, and insecticides, as well as many endogenous lipophilic substrates such as steroids, hemin, and bilirubin. We have found that the "induction," or stimulation, of a group of drug-metabolizing enzyme activities in reponse to aromatic hydrocarbon treatment is regulated by only a few genetic loci in the mouse. We have also employed cell culture techniques for studying the mechanism of microsomal monooxygenase induction in an experimental system which is more rigidly controlled than similar studies in the intact animal. Understanding the mechanism by which drug-metabolizing enzymes are induced may elucidate important concepts in the fields of drug metabolism, developmental pharmacology, steroid biochemistry, chemical carcinogenesis, teratology, entomology, and limnology. The main objectives of this project are (1) to exploit genetic differences in drug metabolism between various inbred strains of mice in order to understand better the potential importance of pharmacogenetics; (2) to use tissue culture techniques in answering questions which would be difficult or impossible with studies in the intact animal; (3) to combine the genetic differences with the tissue culture techniques in order to elucidate certain mechanism related to mammalian gene regulation; and (4) to apply certain of these techniques to human tissues so that extrapolations can be made from rigidly controlled experimental systems to pharmacogenetic differences.

Methods Employed: The principal methods employed are: (1) recording spectrophotometry of turbid solutions, (2) enzyme assays involving radiometric, spectrophotometric, and spectrophotofluorometric determinations of product formation or substrate disappearance, (3) radioisotopic measurements using tritium and carbon-14, (4) cell culture techniques, (5) differential centrifugation, (6) phase microscopy, (7) equilibrium dialysis, (8) polyacrylamide gel electrophoresis, (9) column- and thin-layer chromatography, and (10) magnetic resonance techniques.

Major Findings: (1) Mouse skin tumorigenesis initiated by 7,12-dimethylbenz[a]anthracene or benzo[a]pyrene, with or without promotion by repeated applications of phorbol ester, is unrelated to genetic differences in the extent of aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons in inbred or hybrid C57BL/6N and DBA/2N mice; on the contrary, mouse sarcomas initiated by a single subcutaneous dose of 3-methylcholanthrene is related to the extent of the hydroxylase induction in these parent strains and their progeny from backcrosses and intercrosses. (2) Induction of the microsomal hydroxylase activity by aromatic hydrocarbons is associated with concomitant synthesis of a new CO-binding hemoprotein, "cytochrome P_1450," as determined by (a) spectral changes in the binding of various drugs and the reduced-minus-oxidized difference spectrum, (b) preferential inhibition of benzo[a]pyrene hydroxylation in vitro by various lipophilic test compounds, and (c) paramagnetic changes in oxidized microsomes measured by electron paramagnetic resonance spectroscopy below 10°K; these changes are quite similar in liver or kidney microsomes from mouse, rat, or rabbit. (3) The extent of aryl hydrocarbon hydroxylase induction in various tissues of 10 inbred strains of mice by aromatic hydrocarbon administration in vivo is correlated with the magnitude of hydroxylase induction in fetal cell cultures derived from each of these strains. (4) Cytotoxicity in cell culture, caused by the metabolic potentiation of polycyclic hydrocarbons by the inducible hydroxylase, is correlated with a decreased survival time when aromatic hydrocarbon-"responsive" mice are given large doses of polycyclic hydrocarbons. (5) Aryl hydrocarbon hydroxylase activity also is inducible in more than 5 liver-derived established cell lines treated with polycyclic hydrocarbons, biogenic amines, or phenobarbital in the growth medium. (6) When mice are administred aromatic hydrocarbons, the induction of numerous enzyme "activities"--all membrane-bound monooxygenases having cytochrome P_1450 in their active-sites--is associated with the same genetic locus or with closely linked loci; these enzyme "activities" include: aryl hydrocarbon hydroxylase, p-nitroanisole O-demethylase, 7-ethoxycoumarin O-deethylase, 3-methyl-4-methylaminoazobenzene N-demethylase, zoxazolamine hydroxylase, 2-biphenyl hydroxylase, DT diaphorase, acetanilide hydroxylase, naphthalene hydroxylase, and benzenesulfonanilide hydroxylase. (7) The expression of numerous other microsomal enzymes are *not* associated with this same genetic locus: aminopyrine N-demethylase, NADPH-cytochrome P-450 reductase, NADPH-cytochrome *c* reductase, *d*-benzphetamine N-demethylase, chlorocyclizine N-demethylase, ethylmorphine N-demethylase, pentobarbital hydroxylase, 4-biphenyl hydroxylase, and 6β-, 7α-, and 16α-testosterone hydroxylases. (8) The basal and inducible epoxide hydrase activities are not closely associated with the basal and inducible aryl hydrocarbon hydroxylase activities among various inbred mouse strains. (9) The genetic difference in inducible aryl hydrocarbon hydroxylase activity resides entirely in the fraction containing the CO-binding cytochrome ($P_1$450). (10) A g = 5.95 electron paramagnetic resonance signal is always associated with cytochrome P420 concentration; a g = 8.01 signal height measured below 10° K correlates well with the dithionite reduced-minus-oxidized difference in absorbance between 645 and 700 nm at room temperature. (11) Genetic differences in diphenylhydantoin metabolism between various strains of mice are detectable with an *in vitro* assay and by determining plasma clearance rates. (12) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induces $P_1$450 formation and all the associated monooxygenase activities in so-called "nonresponsive" mice, indicating that these mice probably suffer from a defective receptor binding-site and that they do possess the necessary regulatory and structural genes for the induction process(es). (13) These inducible enzyme activities can segregate as a single autosomal dominant or codominant trait, or the lack of induction can be expressed dominantly, depending on the inbred strains crossed. Mutagenesis *in vitro* by 3-methylcholanthrene, but not by benzo[a]pyrene or 7,12-dimethylbenz-[a]anthracene, as determined by the histidine-tester strains developed by Bruce Ames. (14) The mutagenicity of 3-methylcholanthrene in Salmonella histidine mutant TA1537 or TA1538 *in vitro* is closely associated with the genetically mediated induction of aryl hydrocarbon hydroxylase activity and new cytochrome $P_1$450 formation in the mouse. (15) Threshold differences in the response to 3-methylcholanthrene, 5,6-benzoflavone, or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin have allowed us to distinguish between the homozygote and heterozygote genetically "responsive" mouse. (16) Of the two forms of aryl hydrocarbon hydroxylase activity, only the form associated with cytochrome $P_1$450 is induced in fetal liver or hepatoma cell cultures, regardless of what inducer is used.

Significance to Biomedical Research and the Program of the Institute: Phenobarbital is the most commonly used drug from a class of more than 200 drugs, chemicals, and insecticides that induce microsomal enzymes apparently by the same mechanism. Administration of any of these substances to the pregnant animal causes transplacental induction of these mixed-function oxygenases in the fetal liver. These enzyme systems metabolize steroids, hemin, cholesterol, fatty acids, indoles, sympathomimetic amines, thyroxine, and bilirubin, as well as lipophilic foreign substrates. Administration of polycyclic aromatic hydrocarbons to the pregnant animal produces induction of this enzyme activity in the placenta and also transplacental stimulation of the hydroxylase in several fetal tissues. Cigarette smoking is associated with increases in human placental aryl hydrocarbon hydroxylase activity. Consequently, studies involving the development and extent of induction of mixed-function oxygenases in fetal tissues and in the placenta during various periods of gestation, are relevant to the program of this Institute in the following areas: (1) possible effects of environmental pollutants (i.e., polycyclic hydrocarbons and insecticides) and various drugs on normal fetal growth and development and on the health of the pregnant woman, (2) mechanisms of "pharmacological immaturity" of the premature and newborn, (3) mechanisms of disease of the fetus and newborn concerning oxygenative and conjugative metabolism of normal body substrates such as steroids and bilirubin, and (4) possible therapeutic and diagnostic procedures for aiding and predicting pharmacogenetic defects in the fetus and neonate. As a result of our studies showing genetic differences in aryl hydrocarbon hydroxylase activity in mice, similar differences have now been shown in cultured human lymphocytes; moreover, genetically-mediated high hydroxylase levels have been correlated with bronchogenic and colonic carcinoma in man.

Proposed Course of Project: (1) Further delineation of differences between mixed-function oxygenase induction by phenobarbital and that by polycyclic hydrocarbons, (2) characterization of specific and nonspecific binding of these various inducers to subcellular sites, (3) examination of the effects of hormones and nutrition on hydroxylase induction by either type of inducers, (4) study of various inhibitors on the induction process in cell culture, (5) characterization of the various components of the membrane-bound "hydroxylase" system, (6) analysis of differences in product formation and biophysical properties between the control, phenobarbital, and polycyclic hydrocarbon-induced oxygenases, and (7) extrapolation of these studies to aid in explaining certain mechanisms by which gene expression is controlled in mammalian cells.

Honors and Awards:

Nominated for John W. Abel Award, American Society for Pharmacology and Experimental Therapeutics.

Publications:

1. Nebert, D.W., Considine, N., and Kon, H.: Genetic differences in cytochrome P-450 during induction of mono-oxygenase activities. Drug Metab. Dispos. 1: 231-238, 1973.

2. Benedict, W.F., Considine, N., and Nebert, D.W.: Genetic differences in aryl hydrocarbon hydroxylase induction and benzo[a]pyrene-produced tumorigenesis in the mouse. Mol. Pharmacol. 9: 266-277, 1973.

3. Nebert, D.W.: Use of fetal cell culture as an experimental system for predicting drug metabolism in the intact animal. Clin. Pharmacol. Therap. 14: 693-699, 1973.

4. Nebert, D.W., Considine, N., and Owens, I.S.: Genetic expression of aryl hydrocarbon hydroxylase induction. VI. Control of other aromatic hydrocarbon-inducible mono-oxygenase activities at or near the same genetic locus. Arch. Biochem. Biophys. 157: 148-159, 1973.

5. Oesch, F., Morris, N., Daly, J.W., Gielen, J.E., and Nebert, D.W.: Genetic expression of the induction of epoxide hydrase and aryl hydrocarbon hydroxylase activities in the mouse by phenobarbital or 3-methylcholanthrene. Mol. Pharmacol. 9: 692-696, 1973.

6. Benedict, W.F., Gielen, J.E., Owens, I.S., Niwa, A., and Nebert, D.W.: Aryl hydrocarbon hydroxylase induction in mammalian liver cell culture. IV. Stimulation of the enzyme activity in established cell lines derived from rat or mouse hepatoma and from normal rat liver. *Biochem. Pharmacol*. 22: 2766-2769, 1973.

7. Nebert, D.W., Heidemaw, J.K., Strobel, H.W., and Coon, M.J.: Genetic expression of aryl hydrocarbon hydroxylase induction. Genetic specificity resides in the fraction containing cytochrome P_{448} and P_{450}. *J. Biol. Chem*. 248: 7631-7636, 1973.

8. Nebert, D.W., Robinson, J.R., and Kon, H.: Further studies on genetically mediated differences in monooxygenase activities and spin state of cytochrome P_{450} iron from rabbit, rat, and mouse liver. *J. Biol. Chem*. 248: 7637-7647, 1973.

9. Thompson, E.B., Benedict, W.F., Owens, I.S., and Nebert, D.W.: Aryl hydrocarbon hydroxylase and tyrosine aminotransferase activities in somatic-cell hybrids derived from hepatoma tissue culture HTC (rat) cells and 3T3 (mouse) benzo[a]pyrene-resistant cells. *Biochem. Biophys. Res. Commun*.56: 605-610, 1974.

10. Nebert, D.W., Benedict, W.F., and Kouri, R.E.: Aromatic hydrocarbon-produced tumorigenesis and the genetic differences in aryl hydrocarbon hydroxylase induction. In Ts'o, P.O. and Dipaolo, J.A. (Eds.): *Model Studies in Chemical Carciongenesis*, in press.

11. Nebert, D.W.: Genetic and environmental factors influencing placental and fetal metabolism of drugs. In Moghissi, K.S. (Ed.): *The Placenta: Biological and Clinical Aspects*, in press.

12. Robinson, J.R., and Nebert, D.W.: Genetic expression of aryl hydrocarbon hydroxylase induction. Presence or absence of association with zoxazolamine, diphenylhydantoin, and hexobarbital metabolism. *Mol. Pharmacol*. 10, in press.

13. Poland, A.P., Glover, E., Robinson, J.R., and Nebert, D.W.: Genetic expression of aryl hydrocarbon hydroxylase activity. Induction of monooxygenase activities and cytochrome P_1450 formation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in mice genetically "nonresponsive" to other aromatic hydrocarbons. *J. Biol. Chem*. 249, in press.

14. Robinson, J.R., Considine, N., and Nebert, D.W.: Genetic expression of aryl hydrocabon hydroxylase induction. Evidence for the involvement of other genetic loci. *J. Biol. Chem*. 249, in press.

15. Felton, J.S., and Nebert, D.W.: Carcinogens activated to mutagens *in vitro*: association with genetically mediated increases in aryl hydrocarbon hydroxylase activity and cytochrome P_1450. *Proc. Nat. Acad. Sci. U.S.A*. 71, in press.

NICHD ANNUAL REPORT
July 1, 1973 through June 30, 1974
Pregnancy Research Branch

SUMMARY

The Pregnancy Research Branch has pursued its mission by investigating certain aspects of biochemistry and physiology in normal and abnormal mammalian pregnancy. The animal models used are the pregnant Rhesus monkey, (Macaca mulatta), the pregnant baboon, (Papio papio), and the pregnant Dorset sheep. The fact that data obtained in these preparations equates to that which has become increasingly available from human pregnancy strengthens and supports the belief that extrapolation from these models is pertinent to human pregnancy. Further, it is possible to obtain combined data from an animal pregnancy, whereas it is necessary to collate data from scattered reports in humans.

There are two main groups in the branch. One group has concerned itself with the endocrine physiology that pertains in pregnancy. An ongoing study of carbohydrate metabolism during primate pregnancy has focused particularly on the relationship of fetal and maternal plasma glucagon in the monkey pregnancy and what factors influence these levels. Although both induced hypoglycemia and an alanine infusion to the adult monkey are associated with increments in plasma glucagon, similar stimuli to the intrauterine fetus are not. In contrast, in the newborn there is responsiveness to these glucagonogenic stimuli. Factors that cause this change to occur within the first 12 hours of newborn life are not clear. This lack of responsiveness of the alpha cell is analogous with that found in the pancreatic beta cell.

The renin-angiotensin relationships in sheep pregnancy have been examined. This animal model allows the chronic implantation of both maternal and fetal catheters for periods of up to three weeks and thereby allows the measurement of substances in blood obtained from a nonstressed nonanesthetized state. The injection of the diuretic furosemide into the mother results in a diuresis and an elevation in maternal plasma renin activity. Fetal plasma renin activity does not respond in a consistent way when this is done. Similarly, when the diuretic agent is given directly to the fetus, a diuresis is obtained in the fetus as measured by urinary bladder content but a variable pattern of plasma renin activity is found. In some animals fetal plasma renin activity is markedly elevated but in some there is no change. The reason for this discrepancy at the same time in gestation is not clear. Further, work with fetal nephrectomy as well as the definition of plasma renin concentration and plasma substrate in this model are contemplated.

Examination of calcium homeostasis in monkey pregnancy has been further amplified this year. Parathormone cannot be detected in fetal plasma. When the fetal ionized calcium levels are decreased by the infusion of EDTA directly to the fetus, parathormone is not elaborated. However, although maternal plasma ionized calcium levels do not decrease in this experiment, there is a marked and well defined elevation of maternal serum PTH. The cause of this increment is not clear. When maternal ionized calcium levels are decreased by the infusion of EDTA to the mother, there is no change in fetal serum ionized calcium levels at the same time. Maternal parathormone levels do increase but those of the fetus do not. Newborn plasma PTH levels become detectable anywhere from 12-48 hours of life. When the newborn is challenged with EDTA hypocalcemia from age 6 hours on through 72 hours, there is a prompt increase in serum PTH levels. The cause of the change in response of the parathyroid gland to the stimulus of lowered serum ionized calcium is not clear.

Studies on the amniotic fluid lecithin synthesis in primate pregnancy have been extensively examined this year. The lecithin sphingomyelin ratio abruptly increases at 92-94% gestational age in the monkey as it does in the human. It is possible to examine the individual enzymes activities in the two pathways responsible for lecithin production in the monkey and this has been done both in vivo and in vitro. In both instances radioactively labelled choline and ethanolamine have been used as precursors.

Their incorporation into lecithin has been examined. It is now apparent that there is only one dominant pathway in the monkey and this is the choline pathway. This is consistent with the data now evolving from other animal models including the guinea pig, the rabbit, and the rat. Furthermore, it is now apparent that the original work which suggested two dominant pathways in the human is in error and that the same choline pathway is the one active in the human. The fact that the monkey model is consistent with the data in the human allows the examination of premature induction of the pathway in the monkey with the use of corticosteroids. This work will continue this year.

Fetal experiments in nonhuman primates must be performed under anesthesia and this markedly limits the versatility of the animal model and allows questions of the information obtained from this animal model. This year we have been successful with the maintenance of a chronic preparation in the baboon mother and are now ready to attempt to cannulate the fetal vessels as well. We have been able to improve existing restraining devices which will not compress the abdomen or legs of the mother and at the same time allow chronic cannulation of the carotid vessels to obtain sequential samples of blood in a nonstressed state. We were successful in placing catheters into fetal vessels for a relatively short period of time in one such preparation and have begun examining presently available pharmacologic substance to minimize uterine irritability. This project should result in more success this year.

The second group is primarily concerned with the biochemistry of steroid hormones in primate pregnancy. There is a variety of direct and indirect evidence implicating these hormones in pregnancy maintenance, fetal maturation and the onset of parturition. In addition, assays of their levels in maternal body fluids are used for monitoring feto-placental status in high risk pregnancies.

Using pregnant baboons as models it has been shown that maternal circulating precursor availability is not the only factor controlling estrogen production. Increasing maternal conjugated precursor levels failed to produce proportionate increases in estrogen biosynthesis, an indication that placental capacity is close to saturation in normal pregnancy. However, we have shown previously that variations in the absolute activities of human placental steroid sulfatase and aromatase--two of the key enzymes of estrogen synthesis--do not provide a general mechanism for regulation of estrogen synthesis. Nevertheless, our studies on human term placenta have revealed two potential mechanisms which may serve this purpose. The steroid 3-sulfatase is susceptible to competitive inhibition by endogenous steroids, primarily alternative substrates, and this may regulate estrogen synthesis from conjugated precursors. The 3β-hydroxysteroid dehydrogenase is noncompetitively inhibited by its products of reaction and other Δ^4-3-oxo steroids such as progesterone, which are present in high concentration in placenta and plasma. The latter mechanism would control estrogen synthesis from nonconjugated precursors. Such mechanisms may be exploited to provide for therapeutic control of estrogen production but evaluation of their significance will require experiments with intact cellular preparations and ultimately in vivo.

In order to extend our studies on the importance of precursor availability in regulating estrogen synthesis in baboons we have devised a radioimmunoassay for urinary estrone. This has permitted us to describe the pattern of excretion of this, the principal urinary estrogen during normal gestation. Estrone excretion increases from < 15 μg/24 hr in nonpregnant animals to over 800 μg/24 hr at term. Of greater interest is the 10-fold increase in the rate of estrone excretion during the final 9 days prior to parturition compared with earlier stages of gestation. This is reminiscent of the rise in estriol excretion observed in late human pregnancy. Future studies will utilize this assay to explore the relative importance of conjugated and nonconjugated precursors for estrogen production and the importance of the different placental mechanisms which we suggest may control utilization of each type of precursor.

Studies of cortisol (F) metabolism by pregnant baboons have been greatly expanded. Control experiments with nonpregnant animals showed the degree and type of conjugation, formation of THE and THF as major metabolites and production rate of F to be similar to that reported for humans. Production of highly polar metabolites and extent of side-chain cleavage is, however, greater in baboons. In pregnancy, the proportions of unconjugated and highly polar metabolites increased and glucuronoside formation decreased as they do in humans. The metabolic pattern in postpartum animals was remarkably similar to that in pregnancy. This suggests that metabolic differences between pregnant and nonpregnant animals may reflect changes induced in the mother during pregnancy, and not merely fetal contributions superimposed on unchanged maternal metabolism. Studies with postpartum subjects may prove useful where there is a need to differentiate between fetal and maternal contributions to metabolism during pregnancy. Alterations in the degree of conjugation observed during pregnancy were even more profound in newborn baboons. Since the cortisol binding capacity (CBC) of human maternal serum is known to increase during pregnancy and this may contribute to the alterations in F metabolism at that time it has been measured in baboon sera. Although maternal CBC rises in both species in humans it remains elevated until term, whilst in baboons it decreased towards term but still remains above nonpregnant levels. Whereas fetal baboon CBC at term is 50% of that of the mother's serum, the corresponding value for human infants is 20%. Whether our dramatic finding of elevated CBC in baboon fetal sera at midgestation, compared with values at term, also applies to humans remains to be investigated. As with our studies on estrogen production, elucidation of the mechanisms controlling F metabolism would be greatly facilitated by use of an appropriate animal model since many of the necessary manipulative procedures are precluded in humans. Our current studies continue to reveal sufficient similarities to justify use of the baboon for this purpose.

Our efforts to evaluate and refine existing biochemical methods of assessing feto-placental function in high risk pregnancies have continued. The blood disappearance rates of certain hormones and enzymes advocated as feto-placental function tests showed that the hormones are cleared much more rapidly than the enzymes. The assay of materials with fast disappearance rates theoretically minimizes the time lost between feto-placental compromise and its earliest detection. Thus our findings permit the clinician a rational choice in the method of assessment. Intra-amniotic instillation of dehydroepiandrosterone sulfate (DS) to normal women in the third trimester produced significant increases in maternal blood and urinary estrogen levels. This indicates that in humans the availability of precursors in the fetal environs is a major factor in regulating estrogen production. Since a very rapid response was seen in serum estradiol levels we have proposed that assay of this hormone after intra-amniotic administration of DS may provide a convenient, sensitive and clinically useful index of placental functional capacity.

This branch was considerably strengthened by the addition of more professionals this year and moving some of our laboratories into a new animal operating suite. This animal operating suite constructed to the branch's specifications is available and utilized seven days a week, 24 hours a day. The equipment in the building allows relatively independent function of the unit from the remainder of the reservation. Further, there has been an ample supply of timed gestations in both the sheep and in the monkey during this last year. Unfortunately, the availability of baboon pregnancies has fallen off markedly and efforts are now being directed toward increasing the supply. This relatively large animal with the commensurate increase in size of the fetus as well as its hardiness in stressful situations makes it an important model to examine and to utilize for our continuing experiments in pregnancy.

Occupancy of the clinical facility for this branch will occur this year. Considerable efforts are now being made to attract competent clinicians with research interests oriented toward physiological and biochemical questions in human pregnancy. The staffing of this clinical facility will allow protocols to be applied in the human which both suggest protocols in the animal models as well as allow questions answered in the animal models now to be asked in the human.

Serial No. HD-PR-1
1. Pregnancy Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Carbohydrate Metabolism during Primate Pregnancy

Principal Investigator: Ronald A. Chez, M.D.

Other Investigators: Michael F. Epstein, M.D.; Alan R. Fleischman, M.D.; Gary K. Oakes, M.D.; Daniel H. Mintz, M.D., University of Miami; Antoinette Schenk, University of Miami; Sarah Bowen, B.S.

Cooperating Units: None

Man Years:

Total:	3.0
Professional:	1.5
Other:	1.5

Project Description:

Objectives:

1. To examine the responsiveness of the primate fetal pancreatic alpha cel to glucagonogenic stimuli.
2. To determine if the newborn pancreatic alpha cell responsiveness differs from that of the fetus.
3. To determine if there is a limit to maternal-fetal placental transfer of glucose.

Methods Employed: These have been described last year.

Major Findings: Neither fetal hypoglycemia nor an infusion of alanine directly to the fetus are associated with changes in fetal plasma glucagon levels. This differs from results found when either the mother or the premature newborn are exposed to the same stimuli. In both, there is a prompt elevation of glucagon which is consonant with that found in adult humans. There is no apparent maternal-fetal placental transfer of glucagon. There is a significant linear relationship between maternal and fetal plasma glucose when the mother's plasma glucose levels range from 45 mg % through 1,020 mg %.

Significance to Bio-Medical Research and to the Institute: Evidence continues to accumulate that the pregnant Macaca mulatta is a consistent and reproducible model of those findings that are now being delineated in carbohydrate metabolism of the pregnant human. The model continues to permit the examination of fetal responsiveness to various stimuli, an opportunity which is obviously not available in the human.

Proposed Course: The absence of a chronic fetal preparation in the Rhesus monkey continues to limit the direction of this work. However, the investment has been considerable for the last six years and acute protocols will continue to be applied as information which cannot be obtained in human carbohydrate metabolism is identified. Under consideration at the present time is a protocol directed at the relief of intrauterine growth retardation by the premature induction of fetal beta cell responsiveness to glycemic stimuli.

Honors and Awards: Ronald A. Chez

NIH Medical Board - Member-at-large, 1974.
Member, Council on Resident Education in Ob/Gyn, Committee on In-Training Examinations, 1973.
Invited Lecturer, International Diabetes Federation, 8th Congress, Brussels, Belgium, July, 1973.
Invited Panel Member, Diabetic Pregnancy Study Group, Bruges, Belgium, July, 1973.
Invited Participant, Symposium on the Liberated Woman, New York, New York, October, 1973.
Invited Lecturer, Catholic University, Washington, D.C., October, 1973.
Invited Speaker, Physician/Patient Relations Seminar, New York, New York, October, 1973.
Visiting Professor, Howard University, Washington, D.C., November, 1973.
Visiting Professor, Northwestern University, Chicago, Illinois, November, 1973.
Invited Professor, University of Texas Health Science Center, Dallas, Texas, December, 1973.
Invited Lecturer, Southwestern Gynecologic Assembly, Dallas, Texas, December, 1973.
Visiting Professor, Conference on Menstrual Regulation, Honolulu, Hawaii, December, 1973.
Visiting Professor, University of Montreal, Canada, January, 1974.
Invited Lecturer, Obstetrical Society of Boston, Boston, Massachusetts, February, 1974.
Invited Lecturer, Howard University, Washington, D.C., March, 1974.
Invited Participant, Symposium on Modern Perinatal Medicine, San Diego, California, March, 1974.
Invited Lecturer, Childrens Hospital, Washington, D.C., March, 1974
Invited Lecturer, First International Symposium on Patient-Physician Relationships, New York, New York, May, 1974.
Invited Speaker, American Gynecological Society, Hot Springs, Virginia, May, 1974.
Visiting Professor, George Washington University, Washington, D.C., June, 1974.

Publications:

Salazar, H., Chez, R.A., Pardo, M.: Absence of Ultrastructural Changes in the Basement Membrane of Monkey Muscle Capillaries in Streptozotocin - Induced Carbohydrate Intolerance in Rhesus Monkeys., Amer. J. Patho. 71:437 1973.

Chez, R.A., Reynolds, A.: Fetal Tissue Composition in a Primate Model of the Diabetic Pregnancy., In Fetal and Postnatal Cellular Growth-- The Role of Hormones and Nutrition, Cheek, Wiley-Interscience, New York, 1974, (In Press).

Chez, R.A., Mintz, D.H.: A Monkey Model of Human Pregnancy Complicated by Glucose Intolerance., In Proc. 8th Congress of Int'l Diabetes Fed., Brussels, Belgium, Excerpta Medica, 1974, (In Press).

Gluck, L., Chez, R.A., Kulovish, M.V., Hutchinson, D.L., Niemann, W.H.: Comparison of Phospholipid Indicators of Fetal Lung Maturity in Amniotic Fluid of Monkey (Macaca Mulatta) and Baboon (Papio Papio)., Am. J. Obstet. Gynecol., 1973,(In Press).

Fleischman, A.R., Chez, R.A.: A Chair for the Chronic Study of the Pregnant Baboon., J. Med. Prim., 1974, (In Press).

Reynolds, A., Chez, R.A., Bhuyan, B.K., Neil, G.L.: Placental Transfer of Streptozotocin in the Rhesus Monkey., Diabetes, 1974, (In Press).

Chez, R.A., Mintz, D.L., Epstein, M.F., Fleischman, A.R., Oakes, G.R.: Fetal Glucagon Metabolism in the Subhuman Primate., Amer. J. Obstet. Gynecol., 1974, (In Press).

Chez, R.A., Mintz, D.H., Reynolds, A., Hutchinson, D.L.: Maternal-Fetal Plasma Glucose Relationships in Late Monkey Pregnancy., Amer. J. Obstet. Gynecol., 1974, (In Press).

Chez, R.A., Mintz, D.H., Hutchinson, D.L.: Gastric Glucose Tolerance in the Third Trimester Monkey Fetus., Amer. J. Obstet. Gynecol., 1974, (In Press).

Chez, R.A., Fleischman, A.R.: Fetal Therapeutics - Challenges and Responsibilities., Clin. Pharmacol. & Therapeutics, 14:754-761, 1973.

Chez, R.A.: The Effects of Drugs on the Fetus., Contemp.Ob/Gyn, 2:103-120, 1973.

Andrews, B.F., Apgar, V., Carrington, E.R., Chez, R.A., Finster, M., Freeman, M.G., Gluck, L., Kubarych, S., Queen, J.T., Warrick, L.: Lowering the Risks in Obstetrical Emergencies., Patient Care, 7:20-49, 1973.

Oakes, G.K., Chez, R.A., Morelli, I.C.: Physiological Changes of Normal Pregnancy as Contrasted to Toxemia., Amer. J. Nursing, 1973 (In Press).

Fleischman, A.R., Chez, R.A.: Communicating with the Patient for Optimal Perinatal Care., Contemp. Ob/Gyn, 2:91-93, 1973.

Chez, R.A., Haire, D., Quilligan, E.J., Wingate, M.B., Iffy, L., Rakoff, A.: Status Controversies and Future of Obstetrical Care., In Fogarty Int'l Center Proceedings No. 22, U.S. Gov. Printing Office, 1973 (In Press).

Apgar, V., Chez, R.A., Cohlan, S.O., Conrad, L., Fish, S.A., Greenberg, B.L., Kaplan, N.W., Krugman, S., Mellin, G.W., Myhan, Jr., W.L: Which Drugs for the Pregnant Patient?, Patient Care, 8:54-66, 1974.

Chez, R.A., Perkins, B.B., Love, M.E., Jorgensen, V.: Health Care and the Liberated Woman., Contemp Ob/Gyn, 3:127-152, 1974.

Andrews, B.F., Apgar, V., Chez, R.A., Finster, M., Freeman, M.G., Gluck. L., Queenan, J.T., Warrick, L.: Reducing the Risks of OB Anesthesia., Patient Care, 8:154-174, 1974.

Oakes, G.K., Chez, R.A.: Nutrition During Pregnancy with Particular Emphasis on the Overweight and Underweight Patient, Contemp. Ob/Gyn, 1974, (In Press).

Chez, R.A., Rulin, M.C.: Interdepartmental Sharing of Audiovisual Teaching Aids - A Valid Concept?, Obstet.Gynecol, 43:465, 1974.

Chez, R.A.: Audiovisual Learning Aids in Human Sexuality., Human Sexuality, 1973, (In Press).

Chez, R.A., Mintz, D.H., Hutchinson, D.L.: In Proc. 4th Int'l Congress on Endocrinology, Washington, D.C., Excerpta Medica, 1057-1060, 1974.

Serial No. HD PR 2
1. Pregnancy Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Regulation of Estrogen Synthesis in Pregnant Baboons (Formerly Steroid Hormones in Primate Pregnancy)

Principal Investigator: John D. Townsley, Ph.D.

Other Investigators: C. Deans Crystle, M.D.; Linda J. Gartman; Julie A. Titus, M.S.; Ronald Boardley

Cooperating Units: Experimental Surgery and Clinical Medicine Section, Laboratory Aids Branch, Division of Research Services

Man Years:

Total:	2.0
Professional:	1.0
Other:	1.0

Project Description:

Objectives:

1. To determine the relative importance of maternal circulating precursor availability and placental biosynthetic capacity in regulating estrogen synthesis *in vivo*.
2. To devise a method for estimation of baboon urinary estrone and describe the pattern of excretion of this estrogen during normal gestation.

Methods Employed: Intravenous administration of differentially labelled trace doses of precursor (dehydroepiandrosterone sulfate, DS) and estradiol-17β to animals (Papio papio) in mid and late gestation and subsequent isolation and $^{3}H/^{14}C$ ratio determination of urinary estrone permits determination of the extent of conversion of endogenous DS to estrogen. The method selected for urinary estrone determination was a radioimmunoassay derived from related procedures used in this laboratory.

Major Findings: Increasing maternal circulating DS by administration of nonradioactive material together with the ^{14}C-DS reduced the fraction of DS converted to estrogen. Thus, increasing precursor availability does not result in proportionate increases in estrogen formation showing that in the baboon during normal pregnancy other factors such as placental capacity, in addition to variations in precursor availability, regulate estrogen production.

The estimation procedure for urinary estrone utilizes enzyme hydrolysis and chromatographic purification prior to immunoassay. It has proved satisfactory with respect to specificity, sensitivity, reproducibility and accuracy. For most purposes chromatography can be eliminated since estrone is the predominant estrogen of baboon pregnancy urine. Nonpregnant estrone values were < 15 µg/24 hr; at term they reached mean values of 840 µg/24 hr. Between 70-175 days the rate of increase of urinary estrone excretion was 3.0 µg/day and significantly different from that during the final 9 days of gestation (30 µg/day). This 10-fold rate increase suggests a connection between estrogen production and parturition in this species and is reminiscent of the rise in estriol observed in humans at that time. After delivery, estrone excretion fell rapidly and by 3 days postpartum was close to nonpregnant levels. A similar fall occurred at the time of spontaneous abortion, pointing to the dependence of estrogen production on a functioning fetus and/or placenta.

Significance to Bio-Medical Research and to the Institute: Because of the widespread utilization of estrogen assays to monitor feto-placental status and the apparent importance of such hormones in maintaining pregnancy and in the onset of parturition an understanding of the mechanism controlling their synthesis and metabolism is essential for definitive resolution of many problems of reproductive failure. Our approaches provide fundamental knowledge of these processes which will ultimately be of use to the clinician. Elucidation of controlling mechanisms will require manipulative procedures in both mother and fetus, which are precluded in humans. An adequate animal model must be found to do this and our studies show that the baboon offers great promise for this purpose.

Proposed Course: The availability of a reliable method for estimating baboon urinary estrone excretion and, hence, estrogen production will permit extension of our studies on the role of precursors availability in regulating estrogen production. For example, placental capacity to utilize DS for estrogen synthesis appears limited. Studies with trace doses of radioactive dehydroepiandrosterone (D) and DS indicate preferential conversion of D into estrogen, suggesting limitation of estrogen synthesis from conjugated precursors, possibly by the placental sulfatase. If the more extensive use of D can be confirmed in experiments with mass rather than trace doses of D and DS, this will indicate that variations in D rather than DS availability and placental sulfatase activity may both contribute to regulation of estrogen production. The effects of variations in endogenous production of DS and D will then be examined. There is evidence that fetal estrogen precursors are more readily utilized than those from the maternal circulation. We propose to explore this further by manipulation of the fetus *in utero*.

Honors and Awards: John D. Townsley

Elected to the Perinatal Research Society, 1974.
Member, Ad Hoc Breast Cancer Epidemiology Committee, NCI, NIH, 1973.
Member, Ad Hoc Heart and Lung Project Grant Committee, NHLI, NIH, 1974.

Publications: John D. Townsley

Townsley, J. D.: Further Studies on the Regulation of Human Placental Steroid 3-Sulfatase Activity, Endocr. 93:172, 1973.

Townsley, J. D., Rubin, E. J., Crystle, C. D.: Evaluation of Placental Steroid 3-Sulfatase and Aromatase Activities as Regulators of Estrogen Production, Am. J. Ob. Gyn. 117:345, 1973.

Crystle, C. D., Breuning, F., Dubin, N. H., Grannis, G. F., Stevens, V. C., Townsley, J. D.: Blood Disappearance Rates of Certain Hormones and Enzymes Advocated as Feto-placental Function Tests, Ob. Gyn. 43:41, 1974.

Crystle, C. D., Dubin, N. H., Grannis, G. F., Stevens, V. C., Townsley, J. D.: Investigation of Precursor Availability in the Regulation of Estrogen Synthesis in Normal Human Pregnancy, Ob. Gyn. 42:718, 1973.

Crystle, C. D., Townsley, J. D.: The Effect of Increasing Maternal Dehydroepiandrosterone Sulfate on Estrogen Synthesis by Pregnant Baboons, Endocr. 1974 (In Press).

Townsley, J. D.: Estrogen Excretion of the Pregnant Baboon (Papio papio), Endocr. 1974 (In Press).

Publications: C. Deans Crystle

Crystle, C. D., Sawaya, G., Stevens, V. C.: Effects of Ethinyl Estradiol on Serum Gonadotropins in Postpartum Women, Am. J. Ob. Gyn. 116:616, 1973.

Townsley, J. D., Rubin, E. J., Crystle, C. D.: Evaluation of Placental 3-Sulfatase and Aromatase Activities as Regulators of Estrogen Production in Human Pregnancy, Am. J. Ob. Gyn. 117:345, 1973.

Stevens, V. C., Crystle, C. D.: Effects of Immunization with Hapten Coupled HCG on the Human Menstrual Cycle, Ob. Gyn. 42:485, 1973.

Crystle, C. D., Dubin, N. H., Grannis, G. F., Stevens, V. C., Townsley, J. D.: The Role of Precursor Availability in the Regulation of Estrogen Synthesis in Normal Human Pregnancy, Ob. Gyn. 42:718, 1973.

Crystle, C. D., Breuning, F., Dubin, N. H., Grannis, G. F., Stevens, V. C., Townsley, J. D.: Blood Disappearance Rates of Certain Hormones and Enzymes Advocated as Feto-placental Function Tests, Ob. Gyn. 43:41, 1974.

Crystle, C. D., Hanson, F. W., Stevens, V. C.: Maternal Serum Hormonal Changes in Hypertonic Saline Induced Abortion, Ob. Gyn. 43:447, 1974.

Crystle, C. D., Townsley, J. D.: The Effect of Increasing Maternal Dehydroepiandrosterone Sulfate on Estrogen Synthesis by Pregnant Baboons, Endocr. 1973 (In Press).

Serial No. HD-PR-3
1. Pregnancy Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Amniotic Fluid Lecithin Synthesis in Primate Pregnancy (formerly called Amniotic Fluid Fat Concentrations in Primate Pregnancy)

Principal Investigator: Michael F. Epstein, M.D.

Other Investigators: Ronald A. Chez, M.D.; Alan R. Fleischman, M.D.; Gary K. Oakes, M.D.; Philip M. Farrell, M.D.

Cooperating Units: Pediatric Metabolism Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases.

Man Years:

Total:	5.0
Professional:	4.5
Other:	0.5

Project Description:

Objectives:

To evaluate by *in vivo* and *in vitro* experiments the contributions of pathway I (the choline pathway) and pathway II (the methylation pathway) to the synthesis of lecithin in nonhuman primate fetal lung.

Methods Employed: The *in vivo* experiments are performed approximately 135-145 days of 164 day Rhesus monkey (Macaca mulatta) pregnancy. Cannulation of the mother is via the saphenous vein to the inferior vena cava and the fetus by T-tube of an interplacental vessel. Either C_{14} labelled choline or tritiated labelled ethanolamine are injected into the fetal circulation and sequential samples of fetal blood, maternal blood, and amniotic fluid are obtained over the length of the experiment. The lungs are removed and lung lipids are extracted from the homogenate. The incorporation rates of the two precursors are calculated based on radioisotope counting of the thin layer chromatography separated phospholipids.

Lungs are removed from other fetuses at delivery for the *in vitro* slice work. The lung is immediately iced, sliced by a microtome, and then the tissue is incubated with the same precursors as in the *in vivo* experiment. After suitable time is allowed for incorporation, the lipids in the homogenate are separated. Thin layer chromatography and suitable radio-isotope counting procedures are employed.

Major Findings: Pathway I, the choline incorporation pathway accounts for over 95% of the lecithin found in lung surfactant in the nonhuman primate. The original human work indicating that there were two primary lecithin production pathways involved is not valid.

Significance to Bio-Medical Research and to the Institute: The L/S ratio in human amniotic fluid continues to be a reliable diagnostic tool for the determination of fetal lung maturity. The presence of a mature ratio predicts with considerable accuracy the absence of respiratory distress syndrome in the nursery. Prematurity and particularly respiratory distress syndrome remain the predominant causes of neonatal morbidity and mortality. This animal model now allows research into the physiology and pathophysiology of these pathways in the hopes of eliminating the occurrence of this disease.

Proposed Course: There is evidence that the use of betamethasone given to the human mother before 32 weeks gestation will induce fetal lung maturity. The consistency of this response and the mechanisms by which it works are not clear. There are a total of eight enzymes in the two pathways described for lecithin production. From our preliminary work we believe that it is possible to induce lung maturation in the monkey fetus by the administration of corticosteroids to the mother. The effect of the drug on the kinetics of the individual enzymes involved will be examined.

Honors and Awards: None

Publications: None

Serial No. HD-PR-5
1. Pregnancy Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Calcium Homeostasis in the Subhuman Pregnant Primate

Principal Investigator: Alan R. Fleischman, M.D.

Other Investigators: Ronald A. Chez, M.D.; Gary K. Oakes, M.D.; Michael F. Epstein, M.D.; Daniel H. Mintz, M.D., University of Miami; Samuel Lerman, M.D., University of Miami; Antoinette Schenk, University of Miami; Sally Bowen, B.S.

Cooperating Units: None

Man Years:

Total:	5.0
Professional:	4.0
Other:	1.0

Project Description:

Objectives:

1. To study the effects of perturbations of maternal and fetal serum calcium on the serum calcium, ionized calcium, and immunoreactive parathyroid hormone levels in the fetal and maternal circulations.
2. To investigate the hormonal control of calcium homeostasis in the neonate.

Methods Employed: Rhesus monkeys (Macaca mulatta) with accurately timed gestations are used. Under aseptic surgical conditions, cannulae are placed into the fetal and maternal circulations. An attempt is made to maintain the integrity of the amniotic sac. EDTA is intravenously administered to mother, fetus or newborn. Blood aliquots are sequentially obtained for appropriate measurements utilizing atomic absorption spectrometry, flow through electrodes, and radioimmunoassay.

Major Findings: PTH cannot be detected in fetal serum in the third trimester. When the fetal ionized calcium levels are decreased by the infusion of EDTA, detectable concentrations of PTH are not measured; maternal serum PTH levels do increase. When maternal serum ionized calcium levels are decreased by the infusion of EDTA to the mother, fetal serum ionized calcium levels do not change. There is no detectable concentration of PTH in the fetus.

The serum ionized calcium of the prematurely delivered monkey newborn decreases gradually over the first 24 hours of life. Detectable concentrations of newborn serum PTH are present anywhere from 6-24 hours of life. When the ionized serum calcium is decreased by the infusion of EDTA in the newborn, marked elevations of serum PTH occur promptly.

Significance to Bio-Medical Research and to the Institute: The utilization of the nonhuman primate permits investigation for the first time of the complex relationship between mother and fetus to accomplish mineralization of the fetal skeleton. The lack of responsiveness of the fetal parathyroid gland to an adult stimulus with the gradual change in responsiveness in the first two days of life is consistent with the lack of responsiveness of both the fetal pancreatic beta cell and the fetal pancreatic alpha cell. Mechanisms whereby hormonal responsiveness to usual adult stimuli become active in newborn life are not clear. Newborn tetany and particularly the relationship of hypoglycemia and hypocalcemia in the newborn are areas that remain to be defined.

Proposed Course: No new protocols are planned.

Honors and Awards: None

Publications: None

Serial No. HD-PR-7
1. Pregnancy Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Renin-Angiotensin Relationships in Sheep Pregnancy

Prinicipal Investigator: Gary K. Oakes, M.D.

Other Investigators: Kevin J. Catt, M.D., Reproduction Research Branch, NICHD; Ronald A. Chez, M.D.; Alan R. Fleischman, M.D.; Michael F. Epstein, M.D.; Sarah Bowen, B.S.

Cooperating Units: None

Man Years:

Total:	7.0
Professional:	5.0
Other:	2.0

Project Description:

Objectives:

1. To examine the bi-directional placental transfer of renin.
2. To study the effects of perturbations of maternal and fetal plasma electrolytes and volume on serum renin activity.

Methods Employed: Accurately timed gestations in Dorset sheep are utilized. Under anesthesia and aseptic surgical conditions, cannulae are placed into the maternal and fetal circulations. The latter is done without interrupting the integrity of the amniotic sac. Acute experiments are then performed 3-21 days postoperatively, after the animal has recovered and is in a nonstressed nonanesthetized state. Measurements of renin activity are made utilizing radioimmunoassay techniques.

Major Findings: Potent diuretics (Lasix) administered to the fetus are associated with variable responses in the fetal plasma renin activity. In some fetuses, at 125-135 days, the plasma renin activity is markedly increased. In others who begin with a more elevated basal level, there is no change in the plasma renin activity. The Lasix does result in fetal diuresis as measured by catheterization of the fetal bladder.

Significance to Bio-Medical Research and to the Institute: Factors and variables that regulate and influence the production and secretion of renin in pregnancy are not known. The possibility continues to exist that the system in the fetus and mother are interdependent or independent. It also is possible that fetal plasma renin activity directly affects the maternal placental blood flow and maybe modified or perturbated by fetal needs. The implication of such a relationship may have important human prenatal care in the management of diseased states in pregnancy.

Proposed Course: A fetal nephrectomy at the beginning of the third trimester is a protocol that will be considered to better define the source of plasma renin activity in the fetus, as well as to determine whether maternal renin crosses the placenta. Plasma renin concentration and plasma renin substrate levels will be examined this year as well.

Honors and Awards: None

Publications:

Oakes, G.K., Chez, R.A., Morelli, I.C.: Physiological Changes of Normal Pregnancy as Contrasted to Toxemia, Amer. J. Nursing, 1973, (In Press).

Oakes, G.K., Chez, R.A.: Nutrition During Pregnancy with Particular Emphasis on the Overweight and Underweight Patient, Contemp. Ob/Gyn, 1974, (In Press).

Serial No. HD-PR-8
1. Pregnancy Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Thermo Regulation in the Pregnant Sheep

Principal Investigator: Gary K. Oakes, M.D.

Other Investigators: Adrian Walker, Ph.D.; Ronald A. Chez, M.D.; Michael F. Epstein, M.D.; Alan R. Fleischman, M.D.; Howard D. Metz.

Cooperating Units: Biomedical Engineering and Instrumentation Branch, Division of Research Services

Man Years:	3.0
Professional:	2.5
Other:	0.5

Project Description:

Objectives:

1. To examine the effect of induced maternal hyperthermia on uterine blood flow, placental blood flow, umbilical blood flow, partial pressures of oxygen and carbon dioxide and pH in the blood.
2. To examine the effect of maternal hyperthermia on fetal temperature regulation.

Methods Employed: Pregnant Dorset sheep are operated at 125 days of a 145 day gestation. Electromagnetic flow meters are placed on pertinent fetal and maternal vessels; thermisters and catheters are placed into pertinent maternal and fetal vessels. A chronic preparation is obtained by bringing the leads through a stab incision in the maternal flank. The animal, after a suitable wait for recovery and reestablishment of maternal homeostasis, is placed into an enclosed chamber which allows the control of ambient humidity and temperature. It is possible to raise the maternal sheep temperature to 42° C within 30 minutes by increasing the humidity to about 70% and using radiant reflectors initially. Appropriate measurements are then obtained utilizing the previously placed cannula electrodes, transducers and catheters.

Major Findings: Preliminary data suggest that maternal hyperthermia results in fetal deterioration to fetal death after a relatively short period of time. It would seem that both the uterine and umbilical blood flow cannot keep up with the increase in fetal metabolic requirements.

Significance to Bio-Medical Research and to the Institute: Hyperthermia in human pregnancy may be more of a danger than is presently appreciated. If this work continues to result in significant fetal morbidity and mortality, it may be that some maternal diseases in human pregnancy that are associated with fever are more significant because of the hyperthermia than is presently appreciated. Attempts will be made to induce temperatures which are low grade, spiking, or high grade over several days to better simulate those diseases found in human pregnancy.

Proposed Course: All aspects of the project require exploration and a major commitment to this project is planned.

Honors and Awards: None

Publications: None

Serial No. HD-PR-9
1. Pregnancy Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Placental Mechanisms for Regulation of Steroid Hormone Synthesis in Primate Pregnancy

Principal Investigator: John D. Townsley, Ph.D.

Other Investigators: Elliece Smith, B.S.

Cooperating Units: None

Man Years:

Total: 1.0
Professional: 0.75
Other: 0.25

Project Description:

Objectives:

To elucidate the enzymatic mechanisms which potentially regulate placental estrogen synthesis.

Methods Employed: Radioisotopic kinetic methods were developed to assay in vitro the steroid 3-sulfatase and 3β-hydroxy steroid dehydrogenase of human term placenta. The effects of potential inhibitors were determined from the reduction in initial rate of reaction and the nature of the inhibition by classical kinetic techniques.

Major Findings: Placental enzymatic mechanisms may regulate estrogen synthesis in the presence of excess precursor. It appears that variations in the absolute activities of placental aromatase and steroid 3-sulfatase does not provide a general mechanism for this purpose. However, the placental sulfatase is susceptible to competitive inhibition by physiological concentrations of endogenous steroids and this may provide a mechanism controlling estrogen synthesis. An additional mechanism involving inhibition of placental 3β-hydroxysteroid dehydrogenase by endogenous steroids may also be operative. However, the mechanisms appear quite different. The sulfatase mechanism controls estrogen synthesis from circulating conjugated precursors and the major potential inhibitors are alternative substrates such as pregnenolone and cholesterol sulfates, which are of extraplacental origin. Effective sulfatase levels may be further reduced by progesterone and related steroids formed within the placenta. The product of reaction, dehydroepiandrosterone together with estrogens and intermediates in their synthesis can exert feedback control of their own production by inhibiting the sulfatase further.

All inhibitors examined in detail, whether conjugated or free, appear to act competitively. Thus their effect may be overcome by increasing availability of substrate. The steroid dehydrogenase mechanism, on the other hand, regulates estrogen synthesis from nonconjugated precursors. These precursors are formed within the placenta as a result of sulfatase action or may be derived directly from the circulation, in which case the sulfatase mediated mechanism would be by-passed. Such a by-pass would account for preferential utilization of nonconjugated over conjugated estrogen precursors in placental perfusions in situ and in vitro. The major potential dehydrogenase inhibitors appear to be the product of its action, 4-androstene-3,17-dione, and its analogues including progesterone, all of which originate within the placenta. Alternative substrates and estrogens appear to be of minor inhibitory importance. Since 4-androstene-3,17-dione and progesterone appear to act noncompetitively, additional substrate will not overcome this inhibition, it can only be relieved by further metabolism of the inhibitor and/or its removal from the enzyme environs.

Significance to Bio-Medical Research and to the Institute: Estrogens appear to be important in the maintenance of pregnancy and possibly in the onset of labor. Assays of their levels in maternal body fluids are advocated for monitoring feto-placental status. These studies significantly add to our understanding of the mechanisms regulating estrogen synthesis - an essential factor in resolving many problems of reproductive failure. In addition, they may pave the way to therapeutic control of estrogen synthesis during pregnancy.

Proposed Course: The two potential mechanisms described were derived from studies on disrupted placental preparations in vitro. Further evaluation of their significance will require experiments with intact cellular preparations and ultimately in vivo. At this time, we propose to examine lines of choriocarcinoma grown in culture for their suitability as models for this purpose.

Honors and Awards: None

Publications: John D. Townsley - See HD-PR-2.

Serial No. HD-PR-10
1. Pregnancy Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biochemical Indices of Feto-Placental Status in Human Pregnancy

Principal Investigator: John D. Townsley, Ph.D.

Other Investigators: C. Deans Crystle, M.D.; Norman H. Dubin, Ph.D., University of Maryland; George F. Grannis, Ph.D., and Vernon C. Stevens, Ph.D., Ohio State University

Man Years:

Total:	0.5
Professional:	0.5
Other:	0.

Project Description:

Objectives:

1. To determine the relative puerperal blood disappearance rates of hormones and enzymes purported to be useful in assessing feto-placental status.

2. To assess the influence of precursor availability on estrogen synthesis in late pregnancy.

Methods Employed: Serial blood and urine samples were obtained from normal hospitalized volunteers between 34-36 weeks of pregnancy prior to and after intra-amniotic instillation of dehydroepiandrosterone sulfate (DS) or postpartum. Protein hormones and estradiol-17β were determined by radioimmunoassay, estriol by fluorometry, urinary estrogens colorimetrically and serum enzymes kinetically by procedures developed in this and other laboratories.

Major Findings: Simultaneous serial determinations of serum unconjugated estradiol-17β, total estriol, human chorionic somatomammotropin, heat stable alkaline phosphatase, cystine aminopeptidase and diamine oxidase has demonstrated that the hormones have much shorter disappearance rates (minutes) than the enzymes (days). The use of assays with fast disappearance rates theoretically minimizes the time lost between feto-placental compromise and its earliest detection. Thus, the hormones have a distinct time advantage over the enzymes measured in the present study. Such an asset may be particularly important in following certain high-risk pregnancies where frequent monitoring is required.

Intra-amniotic instillation of DS produced significant increases in maternal serum unconjugated estradiol-17β, total estriol and urinary estrogens, indicating that availability of precursor in the fetal environs is a major factor regulating estrogen synthesis in human pregnancy. The extent of conversion of DS to estrogen appears greater following intra-amniotic than peripheral administration of DS suggesting that precursor of fetal origin may be preferentially utilized. In view of the magnitude (2-5 fold) and rapidity of response in serum unconjugated estradiol-17β to administration of 100-200 mg DS, determination of estradiol-17β 2 hours after intra-amniotic DS administration may prove to be a sensitive and convenient, clinically useful index of placental functional capacity. There were no significant changes in human chorionic gonadotropin or chorionic somatomammotropin when marked elevations in estrogen levels occurred. This implies the absence of a feedback system between these hormones, as measured in maternal peripheral blood.

Significance to Bio-Medical Research and to the Institute: Reliable methods for assessment of feto-placental status are essential in management of high-risk pregnancies. These studies are part of a continuing effort to evaluate and refine existing methods and devise superior procedures for this purpose. The current studies provide a rational basis for selection of the method of assessment (blood enzyme versus hormone assays). The information on precursor availability in regulating estrogen synthesis will facilitate interpretation of data from serum and urinary estrogen measurements in high-risk pregnancies and suggest a new approach to assessment of placental function.

Proposed Course: No protocols are planned at this time because of manpower and clinical material shortages. As these become available it is our priority to pursue development of feto-placental function tests and improvements to existing methods of estimation of urinary estrogens. Our previous studies have shown these to be superior to blood measurements in assessing feto-placental status in most high-risk pregnancies. (See Annual Report 1972-3)

Publications: John D. Townsley - See HD-PR-2

Serial No. HD-PR-11
1. Pregnancy Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Cortisol Metabolism in the Baboon during Pregnancy

Principal Investigators: Gerald J. Pepe, Ph.D., and Robert E. Oakey, Ph.D.

Other Investigators: John D. Townsley, Ph.D., and Nancy Jensen, B.S.

Cooperating Units: Experimental Surgery and Clinical Medicine Section, Laboratory Aids Branch, Division of Research Services

Man Years:

Total:	4.0
Professional:	3.0
Other:	1.0

Project Description:

Objectives:

1. To determine the metabolic fate of circulating cortisol (F) in mature female baboons and compare it with that in humans.
2. To describe the changes in F metabolism induced by pregnancy in the baboon.
3. To compare the metabolism of F by newborn and postpartum baboons.
4. To determine the cortisol binding capacity of serum from maternal and fetal baboons during the course of pregnancy.

Methods Employed: In metabolic studies ^{14}C-F was administered IV to animals maintained in metabolic cages. Urinary extracts were fractionated into unconjugated, glucuronoside and solvolyzable fractions and individual ^{14}C metabolites isolated chromatographically and purified by crystallization and derivative formation. Production rates were estimated from the quantity of ^{14}C associated with urinary tetrahydrocortisol (THF) and tetrahydrocortisone (THE). The cortisol binding capacity (CBC) of serum was estimated from the mass of F bound by serum stripped of endogenous steroids. Equilibrium constants for the interaction between F and baboon sera were measured by dialysis.

Major Findings: After ^{14}C-F administration to nonpregnant animals the rapid rate of excretion (60%/24 hrs., 80%/120 hrs.), type and degree of conjugation (13% unconjugated, 50% glucuronoside, 8% solvolyzable, 20% unextractable), formation of THE and THF as major metabolites, and production rate of F (12 mg/day) were similar to those reported in man. Production of highly polar metabolites and extent of side-chain cleavage, however, is greater in baboons. In pregnancy the proportion of unconjugated and highly polar metabolites was significantly increased while glucuronoside formation and side-chain cleavage decreased. In contrast to nonpregnant animals in which the F/cortisone ratio was < 1, in pregnancy it was > 1. Daily production rates of F and appearance of THE and THF as major metabolites was unaffected by pregnancy. The metabolic pattern of F in postpartum animals was remarkably similar to that in pregnancy. However, its production rate was greatly increased, presumably due to the stress of parturition. The alterations observed in pregnancy were even more profound in newborns. For example, 45% of urinary ^{14}C was unconjugated and only 18% were recovered as glucuronosides.

CBC of maternal serum increased from 33 µg/100 ml (mean values) in nonpregnant animals to 58 µg/100 ml at 60-90 days and fell to 42 µg/100 ml by the end of gestation. At mid-gestation the CBC of fetal serum was indistinguishable from that of maternal serum. However, by term it was only 50% that of the mother. Changes in CBC during pregnancy appear to be due to changes in the concentration of binding protein rather than equilibrium constant, which was similar ($1.3-2.3 \times 10^7$ M-1) for samples from all groups of animals.

Significance to Bio-Medical Research and to the Institute: The importance of intrauterine developmental changes which prepare the fetus for adrenocortical self sufficiency in an extrauterine environment are now beginning to be appreciated. Fetal F may be involved in lung maturation and the onset of parturition as well as homeostatic maintenance during the perinatal period. The production of F during pregnancy is probably related to that of estrogen precursors. The importance of estrogens in pregnancy has been discussed elsewhere (see #HD-PR-2). Elucidation of these processes would be greatly facilitated by use of appropriate animal models since many of the necessary manipulative procedures are precluded in humans. The present study has revealed sufficient similarities to justify use of the baboon as a model. The production rate and metabolic disposition of F and the differences observed in pregnancy and the newborn are similar in many respects to those which can be deduced from isolated studies with humans. As with all models, an awareness of differences is essential to prevent unwarranted extrapolation. The extent of side-chain cleavage and production of high polar materials from F appear to be the most outstanding interspecies differences. Although maternal CBC rises in both species, in humans it remains elevated until term whilst in baboons it decreases towards term but still remains above nonpregnant levels. Whereas the CBC of baboon fetus at term is 50% of that of the mother's serum the corresponding value for human infants is 20%. Whether the dramatic finding of elevated CBC in baboon fetal serum at mid gestation compared with values at term also applies to humans remains to be investigated. Steroids firmly bound to serum proteins are less readily metabolized than nonprotein bound steroids. Changes in CBC may alter rates of F metabolism in both fetal and maternal compartments and in net placental transfer, thereby affecting the growth and function of the fetal adrenal gland during pregnancy. The striking similarity in F metabolism by pregnant and postpartum animals suggests that the differences between pregnant and nonpregnant animals may reflect metabolic changes induced in the mother during pregnancy, and not merely fetal contributions superimposed on unchanged maternal metabolism. This finding also suggests that studies with postpartum subjects, human or nonhuman, may prove useful in other areas where there is a need to differentiate between fetal and maternal contributions to metabolism during pregnancy.

Proposed Course: Studies on F metabolism in newborn baboons will be completed. Attempts will be made to identify a highly polar F metabolite formed in large amounts by neonates and which is different from, but may be related to, the 6β-hydroxycortisol identified in urine from human newborns. Changes in the capability of fetal-newborn baboon tissues to biosynthesize/metabolize F during the perinatal period will be determined. Changes in F concentration in fetal and maternal serum will be determined throughout gestation. Factors responsible for changes in CBC will be investigated. Changes in any of these factors leading to alterations in production rate and/or metabolic clearance rate may be important in affecting fetal maturation.

Publications: Gerald J. Pepe

Morishige, W. K., Pepe, G. J., Rothchild, I.: Serum Luteinizing Hormone, Prolactin, and Progesterone Levels during Pregnancy in the Rat, Endocr. 92:1527, 1973.

Rothchild, I., Billiar, R. B., Kline, I. T., Pepe, G. J.: The Persistence of Progesterone Secretion in Pregnant Rats after Hypophysectomy and Hysterectomy: A Comparison with Pseudopregnant, Deciduomata-Bearing Pseudopregnant, and Lactating Rats, J. Endocr. 57:63, 1973.

Pepe, G. J., Rothchild, I.: Serum Progesterone Levels in Ovariectomized Rats Injected with Progesterone and Estrone: Relation to Pregnancy Maintenance and Growth of Decidual Tissue, Endocr. 93:1193, 1973.

Pepe, G. J., Rothchild, I.: Metabolic Clearance Rate of Progesterone: Comparison between Ovariectomized, Pregnant and Deciduoma-Bearing Pseudopregnant Rats, Endocr. 93:1200, 1973.

Pepe, G. J., Rothchild, I.: A Comparative Study of Serum Progesterone Levels in Pregnancy and in Various Types of Pseudopregnancy in the Rat, Endocr. 1974 (In Press).

Rothchild, I., Pepe, G. J., Morishige, W. K.: Factors Affecting the Dependency on LH in the Regulation of Corpus Luteum Progesterone Secretion in the Rat, Endocr. 1974 (In Press).

Publications: Robert E. Oakey

Oakey, R. E., Heys, R. F.: Differences in 17-oxogenic Steroid Excretion in Pregnancies with Normal and Anencephalic Foetuses, Acta Endocr. 72:156, 1973.

Hancock, K. W., Oakey, R. E.: The Low Incidence of Pregnancy Following Clomiphene Therapy, International J. Fertil. 18:49, 1973.

Hawkins, R. A., Oakey, R. E.: The Estimation of Oestrone Sulphate, Oestradiol-17β and Oestrone in Peripheral Plasma: Concentrations during the Menstrual Cycle and in Men, J. Endocr. 60:3, 1974.

Diver, M. J., Cawood, M. L., Heys, R. F., Oakey, R. E.: The Excretion of 17-deoxycorticosteroids by Women Pregnant with a Normal or an Anencephalic Foetus, Clinical Endocrinol. 2:227, 1973.

Oakey, R. E.: Urinary Excretion of Nonconjugated Oestrogens during the Menstrual Cycle, J. Obstet. Gynecol. Br. Cwlth. 80:794, 1973.

Oakey, R. E., Cawood, M. L., Macdonald, R. R.: Biochemical and Clinical Observations in a Pregnancy with Placental Sulphatase and Other Enzyme Deficiencies, Clinical Endocrinol. 1974 (In Press).

Fraser, H. M., Mitchell, A. J. H., Anderson, C. K., Oakey, R. E.: Interaction of Dehydrotestosterone and Oestradiol-17β with Macromolecules in Human Hyperplastic Prostate Tissue, Acta Endocr. 1974 (In Press).

Oakey, R. E.: Oestrogens in Pregnancy, In The Biochemistry of Women: Methods for Clinical Investigation, Ed. A. S. Curry, CRC Publications, Cleveland (In Press).

Oakey, R. E.: Methods for the Measurement of Oestrogens in Pregnancy Urine, In Methoden der Hormonbestimmung, Eds. H. Breuer and H. L. Kruskemper, Georg Thieme Verlag, Stuttgart 1974 (In Press).

Sewell, P., Wilde, C. E., Oakey, R. E.: Foeto-Placental Function Tests, Ann.Clin. Chem. 1974 (In Press).

Publications: John D. Townsley - See HD-PR-2

NICHD Annual Report
July 1, 1973 through June 30, 1974

Gerontology Research Center

Office of the Chief

Research Services

The Technical Development Section has continued to maintain and service scientific equipment for all investigators at the GRC. In addition to the service functions, the Section has designed and constructed specialized equipment for investigators, such as a complex visual display unit for the Section on Learning and Problem Solving, cyclase assay column holders for the Endocrine Section, oxygen electrode chambers for the Intermediary Metabolism Section, and a motor driven gradient band collector for the Immunology Section.

The on-line capabilities of the GRC computer system have been expanded by the addition of a multi-user digital interface. This interface, designed and constructed locally, is currently being used for on-line pulmonary data reduction. At present approximately 15 different programs regularly use the background capabilities of the computer system to reduce data and perform computations. A manual covering programs developed for the GRC computer has been written to provide detailed information for use of the computer by scientists at the GRC. Programs have been developed for computer analysis of stress electrocardiograms. The detection of ectopic beats is now possible for single-lead electrocardiograms, which make use of the analog and interface capabilities of the GRC computer system. Programs are currently being written to assure proper classification of atypical cardiac rhythms.

The Photography and Arts Section provided drawings, photographic prints, and projection slides as support to 52 GRC scientists and 8 guest scientists working at the Center. The number of drawings provided has increased by 49% to a total of 594. The Section has devised methods for making motion pictures of the circulatory performance in earthworms and the control of cardiovascular activity in monkeys.

The number of animals cared for by the Animal Resources

Facility has increased significantly during the past year. The production colony of the outbred Wistar rats has been maintained at a level of approximately 10,000 animals. The colony is now operating at a mortality rate of approximately 5% per month. During the year, 1400 rats were issued to 12 investigators at the GRC and 3 guest scientists from outside institutions. Of these animals 303 were 24 months of age or older, 472 were 12-23 months of age, and 625 were 11 months and younger. During the year the Animal Resources Facility provided care for 10 rhesus monkeys, 14 guinea pigs, 20 dogs, 62 hamsters, 2,235 rabbits, and 22,150 mice.

In order to meet the needs of the developing programs of the Laboratory of Cellular and Comparative Physiology the number of mice cared for by the Animal Resources Facility is being substantially expanded. It is anticipated that at least 10,000 additional mice, representing 5 different strains, will be added during the coming year.

It has now been demonstrated that the laminar flow tent which was installed last year is as effective as a germ-free room in the rearing of immunosuppressed and immunodeficient animals at a cost which is considerably less than would be required to maintain a germ-free colony.

Inspection of the animal facilities by a representative from the United States Department of Agriculture resulted in a rating of Excellent.

During the coming year installation of a vacuum system for removal of used bedding and waste from the rat and mouse colonies will be installed. Addition of a cage washer to efficiently wash plastic mouse cages will improve the efficiency of the operation.

During the year there was a 62% increase in the number of public inquiries handled by the GRC Public Information Office (268). In addition, there were 110 contacts made through various news media and 223 visitors were provided tours of the facility. In addition, 816 publications and reprints were distributed on the basis of requests made through the GRC Information Office.

Stories dealing with research programs of the GRC were published in the National Observer, Newark Star Ledger, Baltimore Sun Magazine, Baltimore News American, Reader's Digest, Medical World News, and Prevention Magazine.

The GRC Public Information Officer operated a working press room at the Annual Meeting of the Gerontological Society. Many NICHD and NIH grantees had results of their research reported in the 160 press stories that emanated from the meeting.

In addition to providing library services to GRC investigators, the Library staff continued to search out and index the world literature relating to gerontology and geriatrics. During the year over 3,000 references were identified and classified. The reference lists were published quarterly in the Journal of Gerontology.

Collaborative Guest Scientist Program

During the year 18 guest scientist programs were supported by the GRC. Two projects were terminated and 5 new projects were initiated. These programs were staffed by 27 investigators with 21 supporting personnel. The guest scientists programs included studies on age differences in the cardiac performance in the rat, cell biology, gastrointestinal physiology and endocrinology.

Cardiac Performance in the Rat. Isolated heart muscle (left ventricular trabeculae carneae)from hearts of young and old rats has been utilized to identify the mechanisms underlying the prolongation of the duration of contraction of the myocardium of the aged rat under reduced sensitivity to catecholamines with respect to inotropic response of the fold muscle. The major findings from these studies have been the dose response curves to inotropic stimulation with calcium do not change with age. This finding suggests that ability of the contractile element to respond to inotropic stimulation by calcium is not modifed by aging. It has also been found that the age-associated decrease in inotropic response to catecholamines is not due to an increased uptake of norepinephrine by nerve endings, since a similar depression of inotropic response was found in old muscles when stimulated with both norepinephrine, which is taken up by the nerve endings, and isoproterenol, which is not taken up by nerve endings. The decrease in inotropic response to catecholamines thus appears to be related to an age-associated alteration in the response sequence along the catecholamine cascade. This may involve the affinity for content of the catecholamine receptor at the membrane, an alteration in the adenyl cyclase system, or alteration in the calcium storage catecholamine responsive site within the cell. The calcium dosage response curves suggest that the age change in catecholamine response is not related to contractile element responsiveness once calcium mobilization is induced by catecholamines.

In contrast to the altered inotropic response to catecholamines, there was no age associated decrement in the effect of catecholamines on shortening the duration of contraction. The combined effects of the decrease in inotropic response to catecholamines in the equal shortening of the duration of contraction was to markedly decrease the extent to which catecholamine increased the maximal force developed by the old muscle. These results point to the importance of the prolonged duration of contraction of old myocardium as a compensatory mechanism for the decrease in intrinsic contractile ability.

Studies on the electrical and mechanical refractory periods of adult and senile rat myocardium found no age differences in the electrical refractory period but a significant prolongation of the duration of the mechanical refractory period with age was observed. These results suggest that the age-associated prolongation of the duration of contraction is due to alterations in the cardiac relaxing system.

In summary, these studies suggest two major alterations in the mechanical performance of cardiac muscle with age. First, there is prolongation of the duration of contraction which appears to be due to the prolonged active state of cardiac muscle, possibly on the basis of alterations in the cardiac relaxing system. Second, there is a specific age-associated alteration in the inotropic response of cardiac muscle to catecholamines with age.

The direction of future studies will record the measurement of calcium uptake and binding properties of cardiac sarcoplasmic reticulum for comparison with mechanical data. Measurements of the content and affinity of the beta-adrenergic receptor in cardiac muscle from old and young animals, assessment of the elastic and viscous stiffness properties of rat trabeculae carneae during contraction, and, finally, extension of these studies to man insofar as possible by utilizing indirect techniques, namely, with the echo cardiogram.

Cell Biology

When heart slices from adult male rats were incubated with angiotensin a marked stimulation of prostaglandin synthetase was observed. The omission of tyrosine from the incubation medium, however, eliminated the angiotensin-induced stimulation. Rats pretreated with 6-hydroxydopamine had depleted basal levels of prostaglandin synthetase in heart tissue and showed reduced angiotensin-induced stimulation. Norepinephrine also increased prostaglandin synthetase activity in cardiac tissue slices and this effect was not blocked by propranolol. These results suggest that the main mechanism of angiotensin-induced stimulation of myocardial prostaglandin synthesis is through its ability to enhance the release of newly synthetized norepinephrine. These experiments provide a basic model for the study of prostaglandin synthesis and of the mechanism of action in the adult rat myocardium which may be used to explore age-related changes in myocardial performance.

In order to explore further the relationships between cardiac and peripheral vascular function, a study will be undertaken in an attempt to relate calcium pump activity in the vascular smooth muscle to the presence of raised systemic arterial pressure. These studies will utilize the spontaneously hypertensive strain of rats (Okamoto-Aoki). In these experiments the activity of the calcium pump system will be determined in hypertensive animals treated with a combination of reserpine, hydrolysine, and chlorathiazine and control normotensive Wistar rats of different ages.

The fact that cells from different segments of the earthworm have markedly different regenerative capacity makes this animal a useful model for the study of factors involved in cell division and growth. For example, worms cut at the 90th segment will regenerate 10 segments while those cut at the 50th segment will regenerate 40 to 50 segments. Protein fractions isolated from different segments of the earthworm were injected into white New Zealand rabbits and antibodies developed after 6 weeks were used in subsequent tests. Both immunoelectrophoresis and Ouchterlony diffusion immune tests showed that, although the anatomical structure of regions of the worm with very different growth potentials was the same, nevertheless different proteins were identified in worm segments with different capacities for regeneration. It was also shown that the free coelomic cells in the earthworm do not have any appreciable role in regeneration and that the yellow pigment which characterizes many of them is a flavin with an activation maximum of about 345 nm and a fluorescent maximum at about 410 nm. The coelomic cells appear to be centers of lipofuscin deposition.

Attempts to test the Hayflick phenomenon with respect to the number of divisions possible in cells cultured from different segments of the earthworm have encountered technical difficulties in maintaining cells of the earthworm in culture. It has been possible to obtain sterile cultures of cells from wormlings before emergence from the cocoon when they already have their definitive number of segments. Work will continue to define conditions under which cells from regeneration blastemas can be maintained.

It has been found that earthworm blood carries proteins with receptor sites for thyroid compounds T3 and T4. The receptor sites have a high affinity for T3 and T4 but the binding is reversible, indicating that these proteins could be functional in transporting thyroid hormone, although such hormones have not been found in the earthworm.

There is increasing evidence that lysosomes play an important role in cellular metabolism. Lysosomal enzymes are involved in processing and digesting the various macromolecular components of the serum that enter the cell through pinocytosis. In addition, lysosomes may be carriers of hormones in mitogenic substances required for the maintenance of normal cell metabolism and cellular proliferation. Lysosomal enzymes also play a role in the digestion and elimination of worn out or denatured macromolecules and subcellular organelles that takes place in the course of normal cellular turnover. Comparative studies on the properties of lysosomes in transformed cells under *in vivo* and *in vitro* conditions have shown that when human fetal fibroblasts were transferred from *in vivo* to *in vitro* conditions the total specific activities of acid hydrolases and the proportion of unsedimentable enzyme activities were depressed. These results imply a greater lysosomal stability in *in vitro* than *in vivo* conditions. Cytochemically the transfer from *in vivo* to *in vitro* conditions is accompanied by the development of secondary lysosomes, such as residual bodies and multivesicular bodies. The Golgi undergoes hypertrophy and visualization of lysosomal acid phosphatase is enhanced. This change reflects a shift of hydrolases from sites of synthesis and storage to well defined lytic bodies. These changes in lysosomal properties may reflect either stress under *in vitro* growth or may be related to increased pinocytotic activity under culture conditions. It is thus apparent that behavior of lysosomes is strongly influenced by the internal environment and results obtained under *in vitro* conditions must be interpreted with caution.

When transformed lymphocytic cells in culture are treated with vitamin A alcohol a marked increase in the release of lysosomal hydrolases was observed. Autoradiographic studies with labeled vitamin A showed incorporation into many cell structures, including mitochondria, Golgi, and nuclei, which suggests that sublethal and lethal damage induced by the vitamin in these cells alter these structures.

Treatment with vitamin A alcohol also induced changes in the surface coat and charges of transformed lympholytic cells, both in the cell culture and *in vitro* experiments. Treatment with vitamin A resulted in the following changes: (1) Ruthenium Red staining showed a thinner discontinuous surface coat. Positive colloidal iron binding was markedly decreased and particle deposition in sparse clusters separated by extensive gaps. Negative colloidal particle binding was enhanced with the appearance of patchy clusters separated by gaps. These results indicate a loss of surface coat material, reduction in negative cell surface charge, and exposure of positive charges induced by vitamin A. Similar changes were observed in cells treated with neuraminidase, which suggests that release of lysosomal neuraminidase by vitamin A may play a role in the surface changes.

Vitamin A alcohol pretreatment of transformed lymphocytic cells *in vivo* enhanced the antitumor effects of X-radiation. This effect of the vitamin was dose and time dependent. For a given dose of x-rays maximum inhibition of reproductive capacity and survival of the leukemic cells were observed in culture pretreated 4 hours with the vitamin. In combined x-ray and vitamin A treatments maximal inhibition of reproductive capacity and survival as compared with treatment of x-rays alone occurred with a 4-hour vitamin A pretreatment. Since this period of 4 hours coincided with the peak of lysosomal enzyme release induced by the vitamin, the function for cell hydrolases in tumor cell killing by x-rays was postulated. From these results, however, it was not clear whether the effect of vitamin A in tumor cell killing was additive or whether a true sensitization to radiation also took place.

It is possible that the inability of the cultured diploid cell to exhibit the characteristic of immortality possessed by established cell lines may be a reflection of the loss of their ability to incorporate or recognize serum mitogenic factors. In this context it was shown that cultured WI-38 populations exhibited a diminished capacity to absorb macromolecules, presumably including serum mitogens. Further experiments to test this hypothesis are being carried out.

Three components of the adenylate cyclase system have been examined in WI-38 cultured cells in various stages of growth. It has been found that adenylate cyclase activity in late passage cells was almost double that of young passage cells. This suggested that the intracellular level of cyclic AMP should also be increased in the higher passage cells. Initial observations, however, did not support this hypothesis. However, with more precise control over culturing conditions the expected increase in cyclic AMP levels in the high passage cells was demonstrable. Unlike the adenylate cyclase, the cyclic AMP levels decreased in cells at confluence. This suggested that perhaps the phosphodiesterase activity was increasing at confluence and thereby reducing the cyclic AMP levels. However, no such increase could be demonstrated. In fact, there was a reduction in the phosphodiesterase activity at confluence and an increase in phosphodiesterase activity with respect to the higher passage level cells. This problem will be examined in more detail in the coming year.

The kinetics of the uptake of exogenous bovine serum albumin was studied in WI-38 cell populations as a function of culture age (passage). Although the transport constant was the same, the V_{max} for the maximum transport velocity was substantially less in the late passage than in the early passage cells. These results indicate that the metabolic events associated with protein uptake are similar in early and late passage cells, but that the ability to interiorize is diminished in high passage cells.

Exogenously added cyclic AMP has an inhibitory effect on tritiated thymidine incorporation by early passage WI-38 cells as determined by an autoradiographic method. The addition of 1.2×10^{-7} M cyclic AMP slows down the incorporation of ^3H

thymidine such that these cells no longer can be identified as early passage cells. This decrease in percent labeled nuclei from 85% to 55% in cyclic AMP supplemented cells may be a key to control of cell growth and of aging.

In addition to the decrease of DNA synthesis in cyclic AMP treated cells, the rate of protein synthesis (leucine incorporation) was also significantly decreased after exposure of the cells to cyclic AMP. Preliminary studies of RNA synthesis using ^{14}C-uridine incorporation as an index indicated that there is also a slight decrease in the rate of RNA synthesis as a result of cyclic AMP treatment.

Studies have been carried out to determine precisely the localization of polypeptide hormone sensitive adenylate cyclase in cellular organelles of the nephron in the rat. Rat kidney slices were incubated after brief glutaraldehyde fixation in a medium appropriate for the visualization of adenylate cyclase by precipitation of electron-dense lead-phosphate. By electron microscopy the reaction product was observed in the glomerular capillary basement membrane and mesangial matrix, brush border, basement membrane and interdigitating processes in the proximal tubule and basement membranes and the interdigitating processes in the distal tubule. After parathyroidectomy decreased reaction product deposition was noted in the basement membrane and interdigitating processes in the proximal tubule but not elsewhere in the nephron. This decrease was abolished by injection of parathormone after 10 minutes but not after 20 minutes. These findings indicate that parathormone sensitive adenylate cyclase is confined to the proximal tubule, to the basal cell membrane, and its intracellular infoldings.

Effects of Bacterial Enterotoxins

Enterotoxins from human *E. coli* isolated from children with diarrhea from Arizona, Egypt, Tanzania, Yeman, and Czechoslovakia have been identified. Bacteriological and serological results from the Arizona study indicated that about 10% of children with acute nonspecific diarrhea harbored enterotoxigenic *E. coli*.

The adrenal cell tissue culture assay proved to be the most sensitive test yet found for the detection of *E. coli* enterotoxins. This test is based on the observation that *E. coli* enterotoxins stimulate adenyl cyclase in the cells which results in their secretion of steroids into the media and an accompanying morphologic rounding of the cells. Both toxins and antitoxins can be assayed with this method.

Immunological similarities between enterotoxins of *E. coli* and *V. cholerae* were demonstrated by neutralization tests and immunodiffusion studies.

Antitoxin studies indicated that human volunteers infected with enterotoxigenic E. coli developed antitoxin titers during convalescence whereas those infected with invasive strains of E. coli did not.

Rabbits immunized with crude enterotoxin preparations parenterally were protected against homologous enterotoxin challenge in their ileal loops.

Partial purification of the heat labile E. coli enterotoxin has been accomplished by the use of affinity chromatography.

Studies have also been conducted aimed at defining the mechanisms of immunity to intestinal infections. Immunity to cholera has been the model employed. It has been shown that serum IgG antibody traverses the gut mucosa and functions in a protective manner in the gut lumen or at the mucosal surface. In addition, intraintestinal antigen administration is followed by typical local primary and secondary type immune responses with the production of both IgG and IgA antibodies. The secondary response is rapid in onset and could serve to abort infectious processes within the bowel during their incubation period. Protection by active immunization can be inferred either by parenteral immunization or by parenteral priming followed by oral "boosting". Protection by the latter regime may last longer than that following parenteral immunization alone.

These studies indicate that local antigen administration may be an essential component of any immunizing procedure designed to induce local intestinal antibody production. This objective is shown to reproduce the type of immunity which is naturally acquired and age-related and which affords appreciable protection against intestinal infections.

A bacterial substance, cholera toxin, enhances lypolysis in fat cells which is age-related. Young adult rats are much more sensitive to the toxin than are immature or senescent rats. The enterotoxins of both E. coli and V. cholerae also enhance cellular response to epinephrine. The stimulation of lypolysis is due to enhanced adenyl cyclase activity. Epinephrine responsiveness is related to a soluble cytoplasmic protein. The elaboration of this protein is not due to receptor interaction alone since exposure to cholerangenoid, a natural toxoid of the cholera toxins which binds to receptors but does not alter cell function, does not result in the production of the protein. This mediator protein is now being purified and identified in its distribution in tissues other than fat cells.

Motility of Gastrointestinal Tract

Motility of the gastrointestinal tract and its control

by biofeedback techniques. Both fecal incontinence and reflux esophagitis are problems that appear with increasing frequency in an aging population. While symptomatic treatment of reflux esophagitis is generally not difficult, there are some stubborn cases which require surgery, which in itself carries increased risk in the elderly. It is therefore important to develop new nonsurgical techniques for the treatment of these disorders. Additional patients suffering from both fecal incontinence and reflux esophagitis have been successfully treated by using biofeedback techniques. The method has now progressed from an experimental stage to a therapeutic one which is currently being introduced into practice.

Proximal and distal segments of the colon obtained from 6- and 24-month-old rats were used for study of their response to acetylcholine, norepinephrine, secretin, cholecystokinin, and pentagastrin. Isolated in vitro muscle strips suspended in an oxygenated fluid medium were exposed to these agents and contractions of the muscle strip were continuously recorded. Although there was a slight tendency ($p<.04$) for 24-month-old muscle strip to relax more than the 6-month-old strip on exposure to norephinephrine at a concentration of 0.1 μM per liter, the overall dose response curve was not different in the two age groups. There was no significant age difference in response to acetylcholine. There was no evidence of a change in muscle contraction of the strips on exposure to secretin, cholecystokinin, or pentagastrin in either age group.

Bone Mineral Content

Bone mineral content has been assessed in 374 participants in the Baltimore Longitudinal Study (ages 22-94 years) using the Cameron bone mineral scanner which utilizes photon absorptiometry. Reliability of the method has been evaluated by making serial measurements at monthly intervals (6 to 12 months) in 5 normal subjects. Bone mineral content can be estimated within an error of .02-.04 grams of calcium per cm of cortical bone radius. Cross-sectional analysis of the data on 374 normal males yielded a low but statistically significant negative correlation between bone mineral content and age. There was no change in appendicular cortical bone width with age. These data will provide a basis for examining the possibility that osteoporosis prone subjects can be identified prior to the clinical appearance of metabolic bone disease.

Metabolism of Thyroid Hormones

Major emphasis has been placed on studies utilizing isoelectric focusing of intracellular binding proteins and of charged ligands using the LKB Ampholine system.

The Scatchard technique has as yet been unsuccessful in quantitating dog CTBP, as well as rat binding protein (S protein . Thus, no definitive statement about the quantity of cytosol binding proteins for thyroid hormone can be made with regard to age. Qualitatively, however, 2-month-old rats and 24-month-old rats have identical S protein (the carrier of T3 and T4), i.e., electrophoretic mobility, binding site specificity, sex-dependence are identical in young and old animals.

It has also been shown that earthworm blood-coelomic fluid contains a protein which binds T4 with high affinity (estimated 10^6 M^{-1}). Although the species is unable to make iodothyronines, it does iodinate tyrosine to MIT and DIT.

Currently, studies are being directed toward the effect of induced changes in serum free T4 in man on erythrocyte 2,3-diphosphoglycerate. The goal of this study is to assess the metabolic importance of heparin-induced rises in serum T4 on a non-thyroidal enzyme system. As part of this study, changes in 2,3-DPG as a function of age are being monitored.

Intramural Research

The Laboratory of Molecular Aging. The research program on the control of intermediary metabolism focuses on the mechanisms by which metabolic processes are regulated. The thrust of the work is directed towards what we conceive may be the "next frontier" in bio-medical research--membrane biology. The problems and questions studied relate to: (1) the molecular organization of the membrane, (2) the role of the membrane in vectorial transport of small molecular species and proton-motive force, (3) the membranal reception of hormonal stimuli, the transduction and propagation of these stimuli, (4) the catalytic and integrative function of membranes, (5) the turnover of membranes, and (6) the failure to maintain membrane structure, leading to cell death. The fundamental information developed by these studies is essential to and is applicable to the understanding of the biological processes whereby physiological control systems become modified during development and aging.

The uptake of D-glucose into vesicles of membranes isolated from the apical region of the plasma membrane of the renal cortical proximal tubule was shown to be a Na^+-gradient dependent facilitated transport process. In the brushborder system Na^+ gradients were artificially imposed, whereas in more intact systems it is the ouabain sensitive active extrusion of Na^+ across the lateral and basal membranes of the tubule cell that presumably establishes the gradient required for uphill sugar transport. The initial rate of D-glucose transport into the membrane vesicles was stimulated 700% by the Na^+ gradient. The K(stimulation) was 30 mM Na^+. The stimulated uptake was the consequence of a reduction in apparent Km for the sugar and an increase in V_{max}. The uptake of D-glucose in the presence of an initial Na^+ gradient showed an overshoot above the equilibrium value with ultimate return to the equilibrium value as the Na^+ gradient was dissipated during the incubation. Neither Li^+, K^+ nor choline ions caused augmentation of transport. The patterns of inhibition of the Na^+ stimulated transport of D-glucose by sugar analogs and phloridzin closely resembled known physiological data. These findings are consistent with and provide the first strong experimental support in renal membrane systems for the Na^+ gradient uphill transport of D-glucose hypothesized by Crane.

The renal brush border membrane proved to be an effective model for the study of amino acid transport in the kidney. For the first time, the uptake of an amino acid (alanine) by the isolated membrane was shown to be consistent with a concept for a Na^+ gradient dependent facilitated transport of amino acids across the luminal membrane. Na^+ also was found to stimulate γ-glutamyl transpeptidase. This enzyme, whose specific activity was found to be enriched manyfold in the purified brush border preparation, relative to the cortex homogenate, has been proposed recently as the amino acid carrier

Investigations of hormonal regulation of metabolism were focused on the mechanisms of endocrine control of renal transport. Specific questions dealt with (1) the localization in the renal cortex of the membrane bound hormonally stimulated adenyl cyclase, (2) the binding of cAMP and cGMP to luminal membranes, (3) the hydrolysis of cyclic nucleotides by membranal phosphodiesterase, (4) the identification and characterization of the membrane bound protein kinase, (5) the identification of the endogenous protein in the membrane that is phosphorylated, and (6) how the presumed phosphorylated-dephosphorylated protein acts to control the transports of Pi, Ca^{2+}, and perhaps Na^+.

The specific activities of renal cortical adenyl cyclase, basal as well as that activated by NaF, catecholamines and parathyroid hormone, were not elevated significantly in the brush border membrane relative to those in other membranes. This finding suggests that in the renal cortex cAMP is generated on the anti-luminal regions of the plasma membrane. However, since cAMP musttranverse the cell to interact with the luminal membrane, e.g., in the action of parathyroid hormone on Pi transport, and in the appearance of cAMP in the urine, the binding of cAMP to the brush border membrane was characterized. Binding was saturable, approximately 1 pmole/mg protein was bound at 20 nM cAMP, with multiple binding sites. Binding was reversible. Bound cAMP was resistant to hydrolysis by endogenous or exogenous phosphodiesterase. Binding was unaffected by hormones, pH and Na^+ gradients, but was markedly inhibited by sulfhydryl reagents (partially reversed by thiols). cGMP was bound at a rate of 0.1 that of cAMP. cIMP, dibutyryl cAMP and cGMP inhibited cAMP binding.

The levels of cyclic nucleotides at the luminal membrane region of the tubular cell could be controlled by brush border phosphodiesterases. Enzymes hydrolyzing both cAMP and cGMP were evident. At least two enzymes acting on cAMP were found. Phosphodiesterase I was maximally active at pH 8, stable at 53°, apparent Km for cAMP$>10^{-3}$ M, and, upon EGTA inhibition, reactivated by Ca^{2+} but not by Mg^{2+}. Phosphodiesterase II was maximally active at pH 6-7, labile at 53°, and, upon EDTA inhibition, reactived by Mg^{2+} but not by Ca^{2+}. Cobalt activated both chelator-inhibited enzymes.

Protein kinase activity was demonstrated in which the brush border membrane acted as both enzyme and substrate. The apparent Km for ATP was 62 μM and activity was Mg^{2+} dependent (10 mM optimal). The ability of the membrane to phosphorylate itself may be important in control of proximal tubular function.

The role of the membrane in the control of mitochondrial oxidations and, indeed, of the basic mechanisms of energy transduction was studied. These investigations were centered on the control of the complete oxidation of pyruvate, a process involving the operation of both pyruvate dehydrogenase and the entire tricarboxylate cycle, and involved the two main approaches to the regulation of intermediary metabolism: (1) the study of enzyme kinetics _in vitro_, and (2) the variations in the steady state concentrations of key intermediates upon altering the flux through the metabolic pathway.

To pinpoint which of the several reactions of the tricarboxylate cycle in the locus of control in blowfly flight muscle, the steady state concentrations of citrate, isocitrate, α-ketoglutarate, malate, CoA, acetyl CoA, and succinyl CoA were measured in mitochondria during the transition from State 4 (respiration limited by ADP) to State 3 (fully active). On activating respiration with ADP, the mitochondrial contents of citrate, isocitrate, and acetyl CoA decreased whereas those of α-ketoglutarate, succinyl CoA, malate, and CoA increased. Measurements of the tri- and dicarboxylates in the flight muscle of blowflies _in vivo_ before and after initiation of flight were entirely parallel to those found on the activation of mitochondrial metabolism _in vitro_. These changes, combined with the knowledge that the reaction catalyzed by isocitrate dehydrogenase is not at equilibrium, allowed the unequivocal identification of this enzyme as the locus of control in this tissue.

It was found previously that the activity of isocitrate dehydrogenase was inhibited by Ca^{2+}. Thus, Ca^{2+} could effectively function to control the complete oxidation of pyruvate via the tricarboxylate cycle. A kinetic study was carried out on the inhibition of pyruvate oxidation by Ca^{2+}. Pyruvate oxidation was inhibited almost completely by physiological levels of Ca^{2+}; the Ki was 10 μM. The kinetic nature of the inhibition with respect to components of the system, including ADP, Pi and pyruvate, was determined.

The control of pyruvate dehydrogenase activity in isolated rabbit heart mitochondria by a specific kinase and phosphatase, which interconvert phosphorylated (inactive) and dephosphorylated (active) forms of the enzyme was demonstrated.

Dehydrogenase activity was decreased when intact mitochondria were incubated with ATP, oligomycin and NaF. This decrease in dehydrogenase activity was correlated with the incorporation of [γ-^{32}P] ATP into mitochondrial protein and a decrement in the oxidation of pyruvate via the tricarboxylate cycle. Kinase activity was inhibited by pyruvate and Pi and the kinetics of these inhibitions were determined. Inactivation of the dehydrogenase was enhanced by divalent cation ionophores. The inactive phosphorylated dehydrogenase was completely activated by dephosphorylation. The phosphatase was inhibited by NaF and EDTA, and was activated by Mg^{2+}. Reactivation of the dehydrogenase was extremely sensitive to Ca^{2+}, only 10^{-6} to 10^{-7} M was needed for full activity.

Steady state concentrations of intramitochondrial NAD and NADH in flight muscle were measured in order to determine whether the transfer of electrons to the respiratory chain was rate-limiting relative to the transfer of electrons from the respiratory chain to O_2. Intramitochondrial NADH was found to rise on adding ADP to mitochondria oxidizing pyruvate. Experiments in which NAD and NADH were isolated from flight muscle showed that there was a parallel rise in NADH content on initiation of flight. Thus, these results obtained from both the intact insect, *in vivo*, and from isolated mitochondria, *in vitro*, tend to indicate that the activity of the dehydrogenases increase relative to that of electron transport and phosphorylation, on imposition of an energy demand upon the mitochondria. This concept is completely contrary to all other hypotheses on mitochondrial bioenergetics and will be investigated further.

The research program on the control of the expression of genetic information focuses on the interactions of the molecules that are concerned in the propagation of genetic information. The interactions of DNA and RNA with each other, with proteins and with metal ions are studied with the object of understanding these interactions in terms of biological function and particularly in the replication of DNA, the transcription of RNA, and the synthesis of protein, are explored. Synthetic analogs of nucleic acids are used to probe the nature of viral infection and its changes with cellular aging, and to determine how the uptake of foreign macromolecules by cells is affected by cellular aging; it is hoped in this way to produce pharmaceutical agents to offset deleterious age changes. Studies are carried out to elucidate the biological role of important substances like hemoglobin and how structural changes in these substances can lead to aging impairment.

The interaction of metal ions with nucleic acids is of importance because of the requirement of a variety of metal ions in their biological activity and also because of the deleterious influence that environmentally introduced metals can have on nucleic acid structure. This year we employed metal ions for a third purpose: to serve as a probe of the mechanism of transcription of RNA polymerase. We had previously demonstrated the remakably different way in which *cis* and *trans* Pt $(NH_3)_2Cl_2$ react with DNA. The difference in the behavior of these geometrical isomers of a platinum complex was used as a novel means of modifying the DNA template by complex formation involving the different geometries. These experiments lead to the conclusion that the *cis* Pt complex at low concentration and the *trans* complex at higher concentration decreased the length of the message units produced, with the positions of the Pt atoms serving as the message limiting loci. The geometry of the trans complex prevented its interaction with more than one base of the same strand, but the cis complex could readily interact with two adjacent bases, thus restricting their rotation. It appears therefore that RNA polymerase activity stopped whenever such a geometrically restricted base sequence was reached.

In an effort to understand how the binding of proteins to DNA in chromatin depends on the amino acid sequence of the proteins, we studied the interaction of model polypeptides of lysine-rich histones with DNA, and found that different amino acids affected the binding of lysine to the phosphate groups of DNA in different ways. Alanine had very little effect on this binding, whereas tyrosine and phenylalanine had important effects. The placement of amino acid residues between lysines altered the geometry of interactions between lysine and DNA phosphate. The binding of all of these polypeptides to DNA was modified by metal ions.

Through the use of difference ORD spectrophotometry it was shown that poly U message induced a conformational change in the structure of *E. coli* ribosomes. The addition of transfer RNA to the ribosome-message complex had little effect on the rotatory properties.

The thermally induced conformational transitions of single stranded stacked polynucleotides were non-cooperative, *i.e.*, the ordered structure of the molecule broke down gradually. Copper(II) ions made these transitions highly cooperative, *i.e.*, once the ordered structure had been distorted anywhere, it broke down everywhere at once.

It was established that beef pancreatic deoxyribonuclease I, the most frequently used enzyme for splitting DNA, generally split the phosphodiester bond on only one strand adjacent to a given base pair, regardless of the metal ion used to activate the enzyme. These results are in contradiction to the findings from the previously used more indirect methods for studying DNA cleavage.

Initial studies of the effects of metals on (WI-38) cells grown in tissue culture indicated that the concentration of some metal ions was higher in "old" cells than in "young".

Aged cells have decreased ability to protect themselves actively (through the formation of new protein) against viruses; nevertheless, these cells effectively supported the synthesis of viral proteins. During the past year, in an attempt to locate the defects in aged cells we investigated two cellular systems: (1) membrane, for its ability to accept or reject macromolecular material from media, (2) cytoplasm, for production of errors in viral proteins. These aging changes were not sufficient to explain the loss of efficiency of the cells.

The study of vinyl analogs of polynucleotides led to the finding that these substances produce selective toxicity against viral leukemia. To date, this selectivity was detected, in various degrees, in the following systems: (1) Analogs had only a low toxicity to cells in culture and to mice and protected both of them against infection by murine leukemia virus. On the other hand, cells in culture were not protected against several other non-oncogenic viruses. (2) Analogs inhibited rather strongly the reverse transcriptase activity which is characteristic of oncogenic viruses. A detailed investigation of this inhibition revealed that usually only analog-template interactions occurred, but in one single combination of polyadenylic acid with polyvinyladenine the ternary interaction--enzyme-analog-template--was proven.

It was demonstrated that hemoglobin-bound copper directly oxidized the iron in hemoglobin in the absence of oxygen. The copper binding site on the hemoglobin molecule bound other metal ions as well as copper, although only copper produced oxidation. The common binding site for these metals was suggested by competitive binding experiments and a reduction in the rate of copper induced oxidation by the other metals. A possible regulatory role of this metal binding site was suggested by a threefold increase in oxygen affinity when 1 zinc/2 hemes was present.

Laboratory of Cellular and Comparative Physiology. Research activities have continued along the goals set forth a year ago, with particular emphasis on how aging modulates the proliferation and differentiation processes at the cellular and molecular levels. Several model systems are under investigation, ranging from mammals to invertebrates, and the results are discussed beginning with the immune system--the focus of research activities in this laboratory.

Studies on the effects of aging on the immune system are centered about three areas: (a) cellular and functional properties of subpopulations of lymphoid cells, (b) effects of aging on the modulation of proliferation and differentiation of immunocompetent cells, and (c) characterization of artificially induced and naturally occurring immunodeficiency states. During the past year many exciting findings have been obtained in these areas of research and some of them are described in the above order.

A. Cellular and functional properties of subpopulations of lymphoid cells.

1. While there is no appreciable shift in the number and proportion of the major cell types, thymus-derived (T) cells,

bone marrow-derived (B) cells, and accessory (A) cells, in the spleen cells of mice with age, the proportion of the less dense T-mitogen unresponsive cells is increased at the expense of the denser T-mitogen responsive cells. One possibility is that the less dense cells are being selected with age and are less dense due to a change in the nuclear-cytoplasm ratio.

2. The DNA sythetic activity of mitogen stimulated spleen T cells declines with age, but not that of mitogen stimulated spleen B cells, suggesting that there may be an unbalanced shift with age in subpopulations of lymphocytes, and as such a possible unbalanced shift in immune functions.

3. Age-related decline in DNA synthetic activity of T cells is correlated most strikingly with the level of cyclic-GMP. Because changes in the level of cyclic nucleotides can be detected within minutes after mitogen stimulation and long before the onset of DNA synthesis and because there is evidence that changes in the intracellular levels of the cyclic nucleotides initiate and regulate cellular proliferation and differentiation, these results suggest that profound changes may be occurring at the membrane level with age.

4. Subpopulation of spleen lymphocytes have different sensitivity to X-irradiation, mitomycin treatment and freezing rates. This suggests that selective subpopulations of lymphocytes can be prepared through simple treatment of the cells and that the *in vitro* response to antigen or mitogen can be categorized according to what type of cell is responding on the basis of the sensitivity to these various treatments.

B. *Effects of aging on the modulation of proliferation and differentiation of immunocompetent cells*.

1. A humoral nonantigen-specific factor derived from human peripheral lymphocytes is capable of inducing an enhanced antibody response of T cell deficient mouse spleen cells *in vitro*.

2. Thymus cells are normally unresponsive to bacterial lipopolysaccharide (LPS), a B cell-specific mitogen, but treatment of thymus cells with concanavalin A, a T cell mitogen, can influenceLPS-responsive cells to emerge.

3. Thymic epithelial cells of old mice, in contrast to those of young mice, when implanted into T cell-deficient young mice appear to be incapable of transforming precursor cells into functional T cells. Whether or not this age-related defect is irreversible or reversible remains to be resolved.

4. Dietary studies show that (a) perinatal protein undernutrition does not affect the thymus as much as other

organs, suggesting that mechanisms of regulation of its growth may be different from those of other organs, and (b) protein undernutrition of young adult mice can affect both the rate of cellular differentiation and retention of immunologic memory cells.

C. Characterization of artificially induced and naturally occurring immunodeficiency states.

1. Studies on allogeneic chimeric spleens show that they lack a subpopulation of T cells, and the deficiency can be replaced by a nonspecific human lymphocyte factor *in vitro*. A "spin off" of this series of studies is the demonstration that a laminar flow tent is as effective as a germ-free room in the rearing of immunosuppressed and immunodeficient animals (e.g., very old animals) at a cost and effort considerably less than it would take to maintain them in a germ-free room.

2. In autoimmune-prone NZB mice, the cell-mediated immune activity is decreased in all old males, but that of old females was highly variable.

3. Morphological, clinical and immunological analyses of the kidney lesion of Wistar rats revealed that (a) its onset occurs as early as 3 months of age, (b) most of the old rats manifest the lesion (male, 100%; female, 80%), and (c) about 90% of the males and 50% of the females die of the lesion. These results show that these rats are not suitable animals to study aging of certain physiological functions.

Studies of the role of maternal age on the frequency of chromosomal anueploid progenies have been initiated recently with the objective of developing the mouse as an animal model. Encouraging preliminary results have been obtained, e.g., significant numbers of chromosomal aneuploid progenies were observed in retired breeders. In addition, a significant increase in preimplantation death of embryos was noted in the autoimmune prone NZB strain of mouse. Based on these results we are cautiously optimistic that many complex problems on "maternal age effects" in the human can be approached systematically in the mouse.

Research on the mechanisms of *in vitro* cellular aging analyzes what cytokinetic and biochemical events become limiting as different types of human cells age *in vitro*. Three exciting findings have been made. (1) Anueploid cell cultures derived from trisomic-21 patients have a decreased rate of cell replication in comparison to normal diploid cells at the same early passage. This may be responsible for their short "life span". (2) The high RNA content of late passaged WI-38 cell cultures appear to reflect the accumulation of RNA by the non-dividing subpopulation of cells and not to altered RNA synthetic pathways, contrary to the expectation of many. (3) A striking difference in various cytokinetic indices exists between lung and skin fibroblasts obtained from the same embryo. The lung fibroblasts were similar to the early passaged Hayflick WI-38 (lung) cells, whereas those from the skin resembled late passaged WI-38 cells. This discovery emphasizes the danger in equating lung and skin fibroblasts, as done commonly by many, and that caution should be taken in the use of certain in vitro population aging parameters as indices of functional aging at the individual cellular level.

Other studies revealed the following: (1) Massive synthesis of poly(A), which is presumed to have a regulatory role in message metabolism, occurs only during the initial inception of embryogenesis; postfertilization polyadenylation is not altered by inhibitors of DNA-directed RNA synthesis, unlike adult somatic cells; and latent genetic codes are localized in the subribosmal fractions of the ovum, and fertilization rapidly transposes the adenylated genetic messages into the ribosomal fraction. (2) Steroid binding sites in the cytosol appear to decrease with age in various tissues and organs. (3) Rodents can tolerate a diet with a relatively low protein content once they reach adulthood. (4) Age-related changes associated with lysosomal degradation can be detected in papillary myocardial cells.

The Laboratory of Cellular and Comparative Physiology has completed its second year under a new set of goals directed toward an understanding of the effects of aging on regulation of proliferation and differentiation. Associated with the changes in the laboratory goals, there has been a change in research approach to understand biologic mechanisms at the expense of biologic phenomena. This has necessitated a change initiated with the beginning of FY 1974 in the organizational structure of the Laboratory in an attempt to create a scientific atmosphere conducive for productive interaction among the investigators. In view of these changes we are now looking forward to the coming years with great enthusiasm and optimism.

Clinical Physiology Branch. Differences between young and old subjects have been described for a number of hormones. There is, however, very little information on the mechanism underlying these differences. An extensive study of adenylate cyclase has therefore been undertaken in the rat. The studies have proven to be very complex and have opened some interesting areas of cyclase metabolism. First, the hepatic epinephrine-responsive cyclase is increased in senescence but glucagon cyclase does not change. There is some evidence that the mechanism of this difference may reside in age-related alteration of the characteristics of the cell membrane in senescence.

A marked difference in the quantitative and qualitative characteristics of cyclase from homogenates and particulates has been studied. This observation has important implications for the tissue assay of adenylate cyclase generally and for aging specifically. A role for phospholipid in this change is being investigated.

The age-related change of adipose tissue cyclase is being studied in isolated fat cell ghosts. To date the results suggest an age-related loss of *functional* cell membrane *mass* with retention of cyclase in the remaining membrane.

Adenylate cyclase of rat liver and fat cells have been shown to be activated not only by F^-, as previously believed, but by other anions as well ($F > Cl \geq Br > I$). This finding clearly relates cyclase to other anion-activated enzymes. Since a number of such enzymes have been recently studied in pure form these enzymes may serve as models for cylase and provide insight into cyclase action.

The chemistry of renin has been further characterized in the past year. It has been demonstrated for the first time that renin is chemically similar to the "acid" proteases in having an aspartic acid active center (inhibition by diazo-Cu^{++}). Also the kinetics of renin inhibition by pepstatin has been defined. Classical non-competitive inhibition by this most potent inhibitor (10^{-10} M) is similar to our earlier demonstration of non-competitive inhibition by other peptides. Pepstatin was used to estimate renin purity by kinetic means and to calculate renin concentration in plasma.

The studies of the past year were undertaken to explore the functional significance and to establish a mechanism for the age associated changes in intrinisic myocardial contractility. It had been demonstrated during the 1972-73 period that although the total developed tension and rate of tension development were unchanged with age, the duration of contraction was prolonged in aged myocardium, and that the inotropic response to norepinephrine was diminished.

In order to determine whether prolonged contraction duration was secondary to prolonged active state, interventions known to alter active properties were performed. These interventions included exposure to hypoxia and reoxygenation, rapid paired pacing, and measurement of the electrical refractory period. It was found that the action potential duration and effectiveelectrical refractory period in 6- and 26-month-old rats were unchanged with age. However, the old muscles exhibited a significant decrease in the ability to respond mechanically to a second stimulus at short coupling intervals. During recovery from hypoxia, the age difference in contraction duration was markedly accentuated. After tissue catecholamine depletion with 6-hydroxydopamine, the age difference in contraction duration persisted. These findings indicate that increased duration of active state is at least in part responsible for prolonged contraction duration in the aged myocardium and that the age differences in catecholamine content of the muscle are not responsible for the prolonged duration of contraction.

In order to investigate the diminished response to increasing doses of norephinephrine, the dosage response relationship was established for isoproterenol and for calcium. These agents were chosen because their differing inotropic mechanisms allowed insight into the site of the age-associated changes. The response to isoproterenol, which is not taken up by myocardial nerve endings, exhibited age differences identical to norepinephrine. However, the response to calcium which acts directly on the contractile element, was identical in both young adult and aged myocardium. These findings indicate that the contractile proteins of aged myocardium are capable of mounting an equal maximum inotropic response and that the age difference in response to catecholamines lies proximal to the contractile proteins themselves.

Since the inception of the Baltimore Longitudinal Study, the drug, antipyrine, has been administered intravenously to estimate total body water (TBW). The data required for estimate of TBW can also be utilized to estimate plasma half-life (T 1/2) of the drug. In 516 subjects, there is a highly significant decrease in T 1/2 with age. The younger subjects (20-39 years) show a half-life 39% greater than the older subjects (60-79 years). Data on drug kinetics and age are fragmentary and therefore these age differences in T 1/2 of antipyrine, a marker drug for microsomal enzyme degradation, are of great interest.

The past year marked the end of the seventh 2-year cycle of tests in the Longitudinal Study of Human Aging. The completion of Cycle G was chosen as the appropriate time for a comprehensive summarization of our longitudinal data. Summaries on these computer-based data involve a stage of detailed review for completeness and accuracy by a variety of methods. This essential editing has been a herculean task but has now been completed for an extensive array of variables: body composition, bone density, B vitamin study, dietary intake, creatinine clearance, activity and attitudes questionnaire, spirometry, strength and work output, tapping test, nerve conduction velocity, reflex time, reaction time, smoking history, Cornell Medical Index, hearing and vision tests, antipyrine pharmacokinetics, glucose tests, and serum cholesterol and triglyceride concentrations. The increasing prevalence of disease states with age confounds interpretation of physiological age changes. Thus an essential requisite of the comprehensive summary of these data is the classification of subjects on each visit into specific clinical groups. This task has also been completed through Cycle G.

Since it can be anticipated that several of these variables will not be continued into subsequent test cycles, we have also in the past year devoted time to planning future studies on this volunteer population. The following procedures have just been or will soon be added to our study: (1) Echocardiography--a non-invasive technique for evaluation of myocardial function, (2) Sex hormone function (plasma FSH, LH, free and total testosterone levels), (3) Skin fibroblast culture (as a test of the Hayflick phenomenon in man), (4) Post-heparin triglyceride lipase release, (5) Urinary 24-hour sodium excretion (as an index of sodium intake), (6) Retinal photography (age changes have been inadequately studied and also for assessment of a diabetes endpoint), (7) Ethanol infusion study (pharmacokinetics of the drug and cognitive effects as related to age). Several of these new studies involve collaborative efforts with scientists in other laboratories of NICHD, NHLI, and the Johns Hopkins University Departments of Medicine and Ophthalmology.

Analysis of a "tapping test" in 638 subjects showed interesting age differences. This is a paper-and-pencil test in which subjects are asked to tap at two targets which are varied in their size and in their distance apart. The goals include both speed and accuracy. The strategies of individual subjects vary; some attempt to achieve accuracy at the expense of time, while others work rapidly with some sacrifice of accuracy. While aging is associated with loss of both speed and accuracy, the loss in speed averages about 0.8% per year of life while the loss of accuracy was only 0.3% per year. Thus older participants chose strategies which protected accuracy at the cost of increased time to perform the task.

Because of a striking decrease in pyridoxal phosphate (Vitamin B6) plasma levels with age which we previously demonstrated, an addition to our computer program for analysis of the one-week dietary diaries in our participants was written in order to estimate dietary intake of this vitamin. A decreased intake of this vitamin was indeed found--the oldest subjects averaged only 1.27 mg intake/day as compared to 1.77 mg/day for the youngest subjects. These mean values should be compared to the NRC recommended allowance of 2 mg/day. In these 303 subjects, the group who were not taking supplementary vitamin preparations, 6% were taking in less than 1 mg/day and 72% were below the recommended allowance. These data are especially interesting since they are derived from an educationally-advantaged group--the majority of the participants are college graduates.

The detailed activity questionnaire has been partially analyzed. The data are of interest not only from the standpoint of the role of physical activity in aging and disease development, but also from the standpoint of social factors which influence activity patterns. Data are being analyzed for total caloric expenditure and also for intensity of expenditure. Thus activities which require at least 0.06 cal/kg body wt/min (sit-ups, bicycling, swimming, tennis) may result in physiological effects more beneficial than lower level activities even when total caloric expenditure is the same. Age in general results in sharp drop-off in the high energy activities which require socialization (having a partner) and retention of only those activities which can be performed solo (sit-ups). The maintenance of physical activity in the aged would thus be promoted by emphasis on social interaction opportunities. Total activity calories were highly correlated with multiple measures of lean body mass (r values of 0.27 to 0.41, independent of age).

In collaborative studies with scientists at the Johns Hopkins University, two studies on amino acid metabolism in man were carried out. In the first study, the rate of enteric factors in mediating the insulin response to oral amino acids (AA) was studied. AA tolerance tests were performed in obese patients before and 4 months after small bowel bypass. Following surgery 15 inches of jejunum and 5 of terminal ileum were in continuity. A mixture containing 75 mmoles each of leucine, glucine, and lysine were given. The peak increase in immunoreactive insulin (IRI) after AA ingestion was 81 ± 16 μU/ml preoperatively (n = 10). However, the peak increment in AA preoperatively was greater than the post-operative peak increment (Δleu = 775 ± 53 vs. 443 ± 48 μM/L). To prove that the lower postoperative IRI was not due to the lower plasma levels after AA ingestion, 6 additional preoperative subjects were given 40% of the standard AA load. Despite a peak increment in AA (Δleu = 397 ± 69 μM/L) equivalent to that achieved postoperatively, the peak ΔIRI (75 ± 16 μU/ml) was significantly greater ($p < .005$). To show that the pancreatic β cell could respond to AA stimulation postoperatively the standard AA load was given intravenously. The peak Δ IRI was 170 ± 26 μU/ml preoperatively (n = 5). These values are not significantly different. Thus, despite a marked reduction in the β Cell response to oral AA after bypass, IRI release following intravenous AA administration is not impaired. These studies suggest that bypassing the small intestine diminishes the release of an enteric factor(s) which augments the insulin response to oral AA.

In the second collaborative study, the effect of a 60 hour fast and of glucagon on peripheral tissue metabolism was studied. Metabolic effects of glucagon on extrahepatic tissues in man have been postulated based generally on studies employing supraphysiologic glucagon concentrations. In this investigation glucagon was infused into the brachial artery to achieve a physiologic increment in hormone concentration within the forearm and effects on muscle and adipose tissue metabolism sought.

Seven subjects were studied post-absorptively, and seven after a 60 hour fast when the plasma insulin-glucagon ratio was low and increased forearm tissue glucagon sensitivity anticipated. In postabsorptive subjects a 2 hour intra-arterial glucagon infusion (400-600 pg/kg/min) elevated local concentrations by 1156 pg/ml. Glucagon did not alter muscle balance of glucose or amino acids (acidics and neutrals), or adipose tissue glycerol balance. Fasting reduced basal insulin (13.0 to 7.5 μU/ml) and increased glucagon (116 to 135 pg/ml) significantly. Muscle output of eight amino acids increased significantly over post-absorptive values, averaging 87% for threonine, glycine, alanine, methionine, isoleucine, and tyrosine. α-Amminobutyrate release increased 12-fold. Serine, taken up postabsorptively, was released in large amounts. In these fasted subjects the 2 hour glucagon infusion elevated glucagon locally by 748 pg/ml but was without effect on muscle balance of glucose, lactate, amino acids, or acetoacetate. Glucose, lactate, FFA, and glycerol balance across adipose tissue were also unchanged. Local and systemic insulin concentrations were unchanged during all glucagon infusions. Thus, physiologic increments in glucagon are without effect on muscle and adipose tissue metabolism in postabsorptive or 60 hour fasted man. Short term starvation is characterized by a dramatic increase in muscle amino acid release in contrast to the conservation of muscle nitrogen accompanying prolonged starvation.

In an effort to improve the efficiency of routine urinalysis as a screening technique for the presence of renal disease, we have studied the effects of urinary pH, osmolality and centrifugation time on the number of casts seen in the urinary sediment. The effect of each variable was studied independently. In each experiment samples of urine from 6-10 patients with a variety of renal diseases were examined. Counts were performed using a Fuchs-Rosenthal chamber under standard conditions. Each urine specimen was used for the study of only one variable. pH and osmolality were varied within the normal range by the addition of HCl, NaOh, or Na H_2PO_4 and urea or water respectively. There was a highly significant inverse relationship between urinary pH and the number of casts seen. When the data are expressed as the percent of the maximum number of casts seen for each urine, there was a progressive decline from 92%, 81%, 65% and 57% at urinary pH 4.5, 5.4, 6.5, and 7.5 respectively. There was no significant difference in the number of casts counted in specimens centrifuged for 1, 2, 5, 10, 15, or 30 minutes. Likewise there was no effect of osmolality, within the range studied (97-821) on urinary cast counts. Thus, *in vitro* acidification of urine specimens which cannot be examined immediately prevents degradation of casts and increases the likelihood of their identification during routine urinalysis.

The renal response to moderate water deprivation was investigated in 98 participants in the Baltimore Longitudinal Study of Aging. All subjects were free of recent or remote renal disease of any type, diabetes mellitus, coronary or cerebro-vascular disease, urinary tract infection or the administration of medications known to affect renal function. Measurements of urine flow and osmolality were obtained at 3, 6, 9 and 12 hours after the onset of water deprivation. Plasma osmolality was measured at 12 hours. At the onset of the study, subjects were moderately water deprived (urine osmolality 939 ± 26 [SEM] mOsm/L), with no significant age change in urine flow or osmolality. Data from the last six hours of water deprivation showed a significant positive correlation ($R = 0.55$, $p < 0.001$) between age and urine flow, and a significant inverse age relationship with urine osmolality ($R = 0.47$, $p < 0.001$). Since the degree of impairment with age in the ability to decrease urine flow was greater than the relative inability to concentrate urine, osmolar clearance actually increased with age ($R = 0.48$, $p < 0.001$). This age-related impairment in fluid and electrolyte homeostasis may explain the propensity for volume depletion in elderly subjects.

Investigations have begun on a remarkable kindred with familial hemochromatosis, including approximately 80 members in four generations, scattered throughout the middle Atlantic states. This is the largest kindred with familial hemochromatosis to be studied and represents an unusual opportunity to investigate several unresolved questions in this field. A number of studies are performed: oral glucose tolerance, serum iron, liver function tests, 24-hour urinary iron excretion after the administration of a chelating agent, and percutaneous liver biopsy. Light and electron microscopic studies are performed on the biopsy specimens.

Specific emphasis is being placed on determining (1) the natural history of the pre-cirrhotic or latent phase of hemochromatosis, (2) the utility of the serum iron as a screening test for excess iron stores, (3) the influence of alcohol intake on the severity of the hepatic injury from iron excess, and (4) the etiology of the abnormal carbohydrate metabolism associated with hemochromatosis.

Preliminary results of investigations on a portion of the kindred show a high prevalence of excess iron stores (40%), with a pattern of inheritance consistent with an autosomal dominant trait. Serum iron has been found to be an unreliable index of iron stores, and several young adults with repeatedly normal serum irons have been found to have definitely increased hepatic iron. Twenty-four hour urinary iron after desferroximine excretion has not correlated with serum or hepatic iron, and is not useful as a diagnostic test. Thus, liver biopsy seems justified in family members in order to diagnose the disease in an early phase, since venesection therapy can be instituted prior to significant structural damage from iron storage.

Laboratory of Behavioral Sciences. The psychological literature on intellectual function in aging includes a number of references to what is called "the myth of intellectual decline." That phrase is used to mean that intellectual function does not change with age. The evidence for this inference is based on psychometric tests of verbal intelligence which does not decline with age, even among subjects in their 70s and 80s. Research in the Learning and Problem Solving Section during the past year has shown that intellectual functioning that requires the subject to deal with new information does decline with age. A longitudinal study of problem solving showed age deficits in performance among men in their 70s at time of first testing, and age changes (declines) among these same men at retesting. This finding of an age change in dynamic aspects of intellectual function is further buttressed by longitudinal studies of learning and retention. Performance of older men declines markedly when the task requires them to process new information: deficits have been observed in the acquisition , retention and effectiveness of utilization of new information. It seems likely that the difference between these studies of intellectual function and psychometric studies of such function is that the latter tests assess the degree to which subjects can retrieve old, well-learned information whereas our studies assess the ability of subjects to process new information.

In addition to identifying changes in cognitive function with age, we also are discovering important differences in perceptual function with age. In studies of dichotic listening--the appreciation of unique verbal information simultaneously presented to each ear separately--marked age deficits are apparent. Men over 60 have been shown to be unable to report one of two digits, presented dichotically. Younger men have no difficulty recognizing the two digits. The mechanism of this age effect is clearly not related to any changes in memory since men over 60 can easily recall six or more digits when these are presented serially. Several studies in this laboratory have shown that older men are highly susceptible to the effects of interference in learning. It is possible that the dichotic effect may be another manifestation of interference affecting performance in the elderly. If studies now being planned corroborate this interpretation, we will have developed a very powerful tool for assessing the capacity and perhaps even the mechanism by which the brain processes and classifies new information.

In addition to the research on aging in man, there are a number of studies which are looking at basic mechanisms of memory or problem solving. One such study, completed this year, has identified a number of the factors in concept learning which mediate problem solving. One important variable which has been specified in the label which is used to designate examples of the concept: e.g., linguistically positive lables such as yes or no, under some conditions facilitate problem solving. Under other conditions affectively toned labels, e.g., lived or died, facilitate problem solving. There are no conditions under which neutral labels, e.g., A or B, are better than linguistically positive labels.

In addition to the studies described above with man, there also are programs being carried out with lower animals. One study, currently under way, will assess the roles played by socialization, exercise, body weight and food intake on longevity, physical vigor and cognitive function in rats and mice. Results from this project already have shown that paired animals achieve a higher peak of activity than do singly caged animals; and paired animals maintain a higher level of activity after peaking than do single animals.

Last year it was reported that patients with chronic, severe fecal incontinence, secondary to organic lesions could be trained to become continent. It now seems clear, on the basis of continuing clinical studies, that the operant training procedure utilized in last year's research is an important new therapeutic procedure for this bowel disorder. In fact, in light of the dearth of effective alternatives coupled with the ease of training and the high success rate, this method now appears to be the therapy of choice.

Five patients with long-standing essential hypertension were trained to control their systolic blood pressures, i.e., the patients learned to decrease and to increase the level of pressure. Concomitant measures of heart rate (which did not change irrespective of the direction of change of blood pressure) suggest that the patients had learned to modify their peripheral resistances. Three-month follow-up studies of these five patients indicated that they were able to maintain reduced systolic and diastolic pressures (range 5% to 20%), and that they retained the ability to control their systolic pressures.

The autonomic mechanism of learned voluntary control of heart rate in the rhesus monkey has been studied in four animals. The findings are that parasympathetic blockade prevents the animals from speeding their hearts reliably; however, their ability to slow is unimpaired. Sympathetic blockade prevents the animals from slowing heart rate consistently; however, speeding is unaffected. Total autonomic blockade prevents the animals from changing their rates in either direction. Thus, the data indicate that in this preparation heart rate slowing is mediated by withdrawal of β-sympathetic stimulation, and heart rate speeding is mediated by vagal withdrawal.

NICHD ANNUAL REPORT
July 1, 1973 through June 30, 1974
Gerontology Research Center
Laboratory of Behavioral Sciences

In keeping with its role as a laboratory within a research center, the Laboratory of Behavioral Sciences has had a continuing committment to train scientists in aging research as well as to conduct original research. For this reason the laboratory includes among its staff undergraduate and graduate students in psychology as well as post-doctoral fellows. Two of the three permanent, professional staff members have university appointments, and one staff member currently is supervising a graduate student who is working on a doctoral dissertation on a problem related to aging.

The psychological literature on intellectual function in aging includes a number of references to what is called "the myth of intellectual decline." That phrase is used to mean that intellectual function does not change with age. The evidence for this inference is based on psychometric tests of verbal intelligence which does not decline with age, even among subjects in their 70s and 80s. Research in the Learning and Problem Solving Section during the past year has shown that intellectual functioning that requires the subject to deal with new information does decline with age. A longitudinal study of problem solving showed age deficits in performance among men in their 70s at time of first testing, and age changes (declines) among these same men at retesting. This finding of an age change in dynamic aspects of intellectual function is further buttressed by longitudinal studies of learning and retention. Performance of older men declines markedly when the task requires them to process new information: deficits have been observed in the acquisition, retention and effectiveness of utilization of new information. It seems likely that the difference between these studies of intellectual function and psychometric studies of such function is that the latter tests assess the degree to which subjects can retrieve old, well-learned information whereas our studies assess the ability of subjects to process new information.

In addition to identifying changes in cognitive function with age, we also are discovering important differences in perceptual function with age. In studies of dichotic listening -- the appreciation of unique verbal information simultaneously presented to each ear separately -- marked age deficits are apparent. Men over 60 have been shown to be unable to report one of two digits, presented dichotically. Younger men have no difficulty recognizing the two digits. The mechanism of this age effect is clearly not related to any changes in memory since men over 60 can easily recall six or more digits when these are presented serially. Several studies in this laboratory have shown that older men are highly susceptible to the effects of interference in learning. It is possible that the dichotic effect may be another manifestation of interference affecting performance in the elderly. If studies now being planned corroborate this interpretation, we will have developed a very powerful tool for assessing the capacity and perhaps even the mechanism by which the brain processes and classifies new information.

In addition to the research on aging in man there are a number of studies which are looking at basic mechanisms of memory or problem solving. One such study, completed this year, has identified a number of the factors in concept learning which mediate problem solving. One important variable which has been specified is the label which is used to designate examples of the concept: e.g., linguistically positive labels such as yes or no, under some conditions facilitate problem solving. Under other conditions affectively toned labels, e.g., lived or died, facilitate problem solving. There are no conditions under which neutral labels, e.g., A or B, are better than linguistically positive labels.

In addition to the studies described above with man, there also are programs being carried out with lower animals. One study, currently under way, will assess the roles played by socialization, exercise, body weight and food intake on longevity, physical vigor and cognitive function in rats and mice. Results from this project already have shown that paired animals achieve a higher peak of activity than do singly caged animals; and paired animals maintain a higher level of activity after peaking than do single animals.

Last year it was reported that patients with chronic, severe fecal incontinence, secondary to organic lesions could be trained to become continent. It now seems clear, on the basis of continuing clinical studies, that the operant training procedure utilized in last year's research is an important new therapeutic procedure for this bowel disorder. In fact, in light of the dearth of effective alternatives coupled with the ease of training and the high success rate, this method now appears to be the therapy of choice.

Five patients with long-standing essential hypertension were trained to control their systolic blood pressures: i.e., the patients learned to decrease and to increase the level of pressure. Concomitant measures of heart rate (which did not change irrespective of the direction of change of blood pressure) suggest that the patients had learned to modify their peripheral resistances. Three month follow-up studies of these five patients indicated that they were able to maintain reduced systolic and diastolic pressures (range five percent to twenty percent), and that they retained the ability to control their systolic pressures.

The autonomic mechanism of learned voluntary control of heart rate in the rhesus monkey has been studied in four animals. The findings are that parasympathetic blockade prevents the animals from speeding their hearts reliably, however, their ability to slow is unimpaired. Sympathetic blockade prevents the animals from slowing heart rate consistently, however, speeding is unaffected. Total autonomic blockade prevents the animals from changing their rates in either direction. Thus, the data indicate that in this preparation heart rate slowing is mediated by withdrawal of beta-sympathetic stimulation, and heart rate speeding is mediated by vagal withdrawal.

Serial No. HD-LBS-1
1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Physiological Psychology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Behavioral genetics and aging

Previous Serial Number: Same

Principal Investigator: Charles L. Goodrick, Ph.D. (40%)

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	.80
Professional:	.40
Others	.40

Objectives: The principal objectives of this project are (1) to determine group differences for behavioral traits and longevity among inbred strains of mice; (2) to determine: (a) heritability (degree of genetic determinatio vs. degree of environmental determination); (b) mode of inheritance (e.g., over-dominant, dominant, intermediate, or recessive); and (c) number of segregating units (gene blocks) controlling a particular trait (e.g., longevity); and (3) to examine relative behavioral differences among mouse strains as aging progresses.

Other objectives include analyses of the influence of diet (e.g., protein available) on behavioral traits, growth, and longevity; and determination of single-gene influences upon behavioral traits, growth, and longevity.

Methods Employed: Inbred mice (C57BL/6J and A/J) of a high degree of homozygosity are maintained under uniform environmental conditions. The animals are tested behaviorally during one period of their life span, e.g., when mature, mature-old, or aged. Old age is determined as the 50% mortality point for groups maintained throughout their life span. Statistically reliable techniques have been developed to determine behavior relevant to natural selection such as exploration, general activity level, emotionality, simple or complex problem solving ability and taste preference. The use of segregating groups allows an estimate of the mode of inheritance, e.g., dominant or intermediate, and the number of gene blocks or segregating units controlling behavioral traits or life span. The diets used for studies in which protein intake is varied among groups of inbred and hybrid mice are isocaloric synthetic diets obtained from General Biochemicals, Chagrin Falls, Ohio. Deprived animals receive 4% casein in their diets whereas the control group receives a 26% casein diet. Numerous kinds of mutant mice are maintained on the C57BL/6J background at the Jackson Memorial Laboratories; Bar Harbor, Maine. Our work has concentrated on the albino, beige, yellow and obese mutations.

Major Findings:

A. An experiment has been completed to determine the effects of aging and genetic constitution upon behavior and longevity for A/J and C57BL/6J inbred mice. A/J mice are albino (white coat), short lived, behaviorally inactive mice, while C57BL/6J mice are melanic (black coat), long lived, behaviorally active mice. Both groups die of nonspecific causes. Groups of A/J, C57BL/6J, F_1 and F_2 mice have been tested on a number of behavioral measures with an N at least 16 per group at 5, 14, and 23 months of age, and at the time of advanced old age for the four groups: A/J (23 Mo.) C57BL/6J (26 mo.), F_1 and F_2 (29 mo) at the approximate time of senescence or 50% mortality. Behavioral data were collected using standard tests developed in our laboratory to determine: (1) sensory discrimination, (2) preference for ethyl alcohol, (3) performance and retention of a learned operant response, (4) exploration behavior, and (5) emotionality.

Measures of open field exploration have been analyzed and are given in Table 1. The normal mode of inheritance is intermediate at 5, 14, and 23 months of age. In general, strain differences are reduced with increasing age. At senescence, strain differences were at the lowest level on the last two exploration trials. The coefficients of genetic determination (Table 2) were consistently high over trials for all age groups, a finding consistent with previously published research from our laboratory using only mature-young mice. However, with respect to age differences, the coefficient of genetic determination was reduced only for the populations examined at senescence. The estimates of the number of loci also were similar to those obtained in previously published research using mature-young groups. Although number of genetic loci were estimated as 5 to 6 on trial 1 for mature-young mice; by the third test trial, the number of genetic loci which affect exploration behavior was found to be one. A major progressive age difference was obtained in the reduction of the number of loci affecting exploration behavior as aging progresses, at least on the initial test trial.

B. A number of groups of mice currently are being maintained to obtain mortality data. Our early studies used A/J, C57BL/6J, F_1, and F_2 groups for studies of behavioral differences for populations which differ with respect to genetic constitution and age. In addition to behavioral measures, 100 animals for each group were used to determine average longevity. In order to obtain additional information and determine the possible generality of our earlier findings, we are maintaining populations of A/J, Balb/cJ, C57BL/6J, and DBA/6J inbred mice, and the six possible F_1 hybrid groups. This study will yield information with respect to longevity of these inbred strains, and additional information with respect to the mode of inheritance of longevity. Groups of four mouse mutations with littermate controls also are being maintained to determine longevity, all on the C57BL/6J background. These four mutations (Bg, A, Y, and ob) all differ from controls on the major dimension of body weight. Further data also are being obtained with regard to the body weight and longevity of A/J, C57BL/6J and F_1 hybrid groups fed isocaloric synthetic diets which differ in protein content. These longevity projects will continue for an additional 24 months.

Table 1

Mean scores of exploration activity as a function of age and trials.

TRIALS

	1	2	3
5 mo. old			
C57	50.2	32.1	28.2
A/J	6.9	8.7	7.6
F_1	27.8	24.7	21.8
MP[a]	28.6	21.4	17.9
14 mo. old			
C57	46.0	41.5	28.2
A/J	9.0	8.0	10.1
F_1	26.3	23.0	19.8
MP[a]	27.5	24.8	16.1
23 mo. old			
C57	42.7	32.4	25.8
A/J	14.1	12.6	12.0
F_1	20.	21.1	16.2
MP[a]	25.3	19.4	15.8
Senescence			
C57	40.3	20.3	16.4
A/J	14.1	12.6	12.0
F_1	26.1	15.6	12.5
MP[a]	27.2	16.4	14.3

[a] MP = Mid-parental values

Table 2

Genetic determination (h^2) and estimates of No. of loci (K) for exploration activity as a function of age and trials.

Trials	h^2	k
5 mo. old		
1	.604	5.52
2	.470	2.85
3	.610	0.71
14 mo. old		
1	.636	2.69
2	.411	3.15
3	.629	0.61
23 mo. old		
1	.572	1.45
2	.439	1.19
3	.704	0.31
Senescence		
1	.418	1.31
2	.301	0.22
3	.358	0.75

Significance to Bio-Medical Research and Program for the Institute: The study of the genetics of behavior and longevity allows an assessment of: (1) the mode of inheritance for the factor studied (i.e., dominant, intermediate, etc.); (2) the relative importance of hereditary and environmental factors; and (3) the number of genes or gene blocks which control the factor studied. Lack of adequate dietary protein is a condition which affects a large proportion of the world populations. Our program attempts to determine the effect of diet (such as different proportions of protein in the total diet), during particular stages of the life span upon behavior and longevity for animal populations which differ in genetic constitution. Studies of single gene mutant animals are of importance because they allow the assessment of the importance of a specific genetic locus for physiological or behavioral factors.

Proposed Course of Project: Further inbred strains and F_1 hybrid groups are being studied to determine the generality of mode of inheritance for behavioral factors. Cross-sectional and longitudinal studies of mouse behavior will continue with mouse strains BALB/cJ and DBA/2J, in addition to the A/J and C57BL/6J mouse strains. The longevity of inbred and hybrid groups are also being determined. Experiments with low and normal protein diets should determine: (1) the relationships between growth rate and longevity, (2) the effect of low, normal, or high protein diets upon behavior at maturity after access to these diets during various stages of development, and (3) the effects of a present diet of low, normal, or high protein upon behavior.

Honors and Awards: None

Publications:

Goodrick, C. Exploration Activity and emotionality of albino and pigmented mice: Inheritance and the effects of test illumination. Journal of Comparative and Physiological Psychology, 1973, 84, 73-81.

Goodrick, C. The effect of dietary protein upon growth of inbred and hybrid mice. Growth, 1973, 37, 355-367.

Goodrick, C. The effect of protein malnourishment and caging on growth, exploration activity, and emotionality of inbred and hybrid mice. Developmental Psychobiology, In Press.

Serial No. HD-LBS-2
1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Physiological Psychology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Daydreaming and Aging: Normative and Experimental

Previous Serial Number: Same

Principal Investigator: Leonard M. Giambra, Ph.D. (50%)

Other Investigators: Judy S. Ruth, B.A.
Mark Filler, B.A.
Jerome L. Singer, Ph.D.

Cooperating Units: Baltimore City Hospitals
Community College of Baltimore
Loyola College of Baltimore
College of Notre Dame of Maryland
Miami University of Ohio
Bykota Senior Citizen's Center of Baltimore
Division of Computer Research and Technology, NIH.

Man Years:

Total:	.60
Professional:	.50
Others:	.10

Project Description:

Objectives:

1. To determine the incidence and content of daydreaming in the population in general and in specific subpopulations, e.g., young, middle-aged, elderly, from various socio-economic classes, various races, etc;

2. To attempt to relate these differences in daydreaming to any underlying mechanisms such as physiological state, education, sub-cultural values and beliefs, differential daily experiences, etc;

3. To investigate experimentally variables which normative studies have indicated may be potent determiners of daydreaming.

Methods Employed: The normative aspects of daydreaming are determined through the use of structured self-report. Each participant completes a 21 item biographical questionnaire which has both specific and general items concerning daydreams, night dreams, fantasy, etc. To date approximately

700 individuals from a wide variety of subpopulations have completed the IPI and bio-questionnaire. The experimental examination of daydreaming has been accomplished through the use of polygraphs to record eyemovements, and through stimulus presentation devices which have been used to control the rate and kind of stimuli that an individual must deal with in order to determine daydreaming propensity under these controlled conditions. There are 28 scales in the IPI. Each scale, except Scale 3, is based upon the responses to 12 items--Scale 3 involves 20 items. No item is used on more than a single scale. Not all subjects completed the IPI but whenever all the items of a scale were completed a value for that scale was computed. Therefore, the number of subjects in each age group for whom a scale's value was obtained varied from scale to scale. Each item has five alternatives which are points on a continuum implying frequency or quantity. The alternatives were assigned values of 0, 1, 2, 3, or 4.

Major Findings:

I. Daydreaming as a function of age.

Seven age groups were used: 17-23 (n = 214), 24-34 (n = 20), 35-44 (n = 13), 45-54 (n = 31), 55-64 (n = 46), 65-74 (n = 28), and 75-91 (n = 26). The youngest age group consisted of students of Miami University. The remaining groups were composed of participants in the Baltimore Longitudinal Study. All subjects were male. The dominant majority were middle or upper-middle class whites. The youngest age group nearly all had one or two years of college education. The older age groups had 10% with less than a college education, 10% with 1-3 years of college, 25% with 4 years of college, and 55% with more than 4 years of college. All subjects were volunteers and were included if they volunteered.

Daydreaming was shown to be a function of age (see Table 1). With increasing age a linear decline in daydreaming frequency was observed. In addition, for daydreamers of equal frequency a linear decline with increasing age was noted for scales reflecting: (a) absorption in daydreaming, (b) mental habits such as mindwandering, boredom, distractibility, and need for external stimulation, (c) future temporal settings, (d) affective reaction in daydreaming, (e) imagery--especially auditory--and vividness of daydreaming, and (f) content such as sexual, bizarre-improbable, achievement-oriented, hostile, heroic, fear of failure, and guilt. No decline with age was noted for acceptance of daydreaming as normal, impersonal and interpersonal curiosity, mentation rate, present and past orientation of daydreams, and problem solving daydreams. Impersonal curiosity and past orientation showed a small tendency to increase with age. Differences between mean values of the scales reflecting temporal setting of daydreams for any age group were not greater than 5.3. This indicated that all temporal settings were about equally important at every age. Relative mean scale values showed present orientation as strongest for the middle span of years, 24-64, while future orientation was strongest for the youngest span and past orientation strongest for the 65-91 years of age span. The change in the relative importance of the temporal orientations fits well into our folk knowledge of aging; however, the closeness does not (see Table 1).

The great weight given to problem solving daydreams previously reported by others also was found in this study and was shown to extend to age 91. These findings provide strongest support for the notion of fantasy as reflecting the current concern of the individual and as involving a large component of information processing and reorganization. Sexual daydreams showed an age related decline even for daydreamers of equal frequency. Furthermore, sexual daydreams moved from top rank for the youngest group to second rank for the next four age groups to third and fourth rank for the two oldest groups. All daydreaming content except problem solving moved to minimal importance and occupancy of waking time with increasing age. One might say that the 75-91 age span involved essentially problem solving daydreams.

One investigator (JLS) has shown that daydreaming and personality in college populations may be viewed as composed of four factors or dimensions. One factor was "Neuroticism-Anxious, Poorly Controlled Thought". When this factor was compared with the factor structure for this study (see Table 2) it was nearly identical to Factor III. Age was the sole occupant of the negative pole of Factor III. Thus neurotic-anxious, poorly controlled thought as expressed by mindwandering, boredom, daydreaming frequency, distractibility, sexual daydreams, and absorption in daydreams played a smaller and smaller part in mental life with Ss who were older and older. One might interpret Factor III as indicating that pathological aspects of fantasy decline with age. This interpretation must be viewed cautiously since it is not based on a longitudinal study; the older age groups might have been composed of fewer pathological subjects by virtue of survival and ability to participate in the study.

The second factor was "Obsessional-Emotional Daydreaming" and was nearly identical to Factor I of this study. Age alone was the negative pole of Factor I. Obsessional-emotional daydreaming as expressed by content involving guilt, fear of failure, hostile acts, achievement, heroic acts and by frightened reactions and vividness played a decreasing part in the daydreaming activity as age increased. This factor seems to represent an "action-intensity" dimension to daydreaming. The decline of action and intensity in daydreaming with increased age is seen as representative of a diminishing desire and capacity for both with age. The older one becomes, the less guilt, fear of failure, heroic actions, and achievement he daydreams about because he is more and more likely to have been through it all or because it no longer concerns him.

The third factor was "Positive-Vivid Daydreaming". This factor was very similar to Factor VII found in this study. Age did not load in this factor. Thus there is an aspect of daydreaming involving vividness, imagery, and absorption about sexual activities, the past, and the solving of problems leading to a positive reaction which was true for every age group studied.

The fourth and final factor of daydreaming previously reported and also replicated by this study was called "Controlled Thoughtfulness". Factor VI in this study was most like Controlled-Thoughtfulness. Age also did not load on this factor. Factor VI showed an aspect of mental life centered around curiosity, with acceptance of and positive feeling toward daydreams of the future and achievement of problem solving. This factor showed that there was an ingredient in the lives of subjects of all age groups built around a pattern of daydreaming and mental activity which was controlled and reflective.

II. Eyemovements as a function of attention and rate of change in thought content.

Reduced eye movement (EM) rate has been related to entertaining daydream-like thoughts, to imagining stationary objects, to maintaining a passive state of mind, and to detection of auditory signals. Increased EM rate has been related to conscious suppression of specific thoughts, to maintaining an active state of mind, and to imagining moving objects. Thus a positive relationship can be inferred between change rate of cognitive content and EM rate. Some researchers have contended that the positive relationship is between attention level and EM rate. This alternate position is based upon an alertness model in which higher attention leads to higher overall arousal which in turn leads to increased EM rate. EM rates have been found to increase during a word association task and during mental multiplication. Since attention and thought content change were confounded in previous studies they could be interpreted as supporting either hypothesis. The confound was removed in this study which investigated EM rate under conditions of high and normal attention while engaged in a task involving few and many changes in thought content.

A 2 × 2 factorial design was used. The factors were attention levels (high, normal) and rate of change of thought content (high, low). Six male and six female volunteers from introductory psychology class participated at each change rate of thought content. Subjects were exposed to both attention levels. The two attention conditions were manipulated by task and instructions.

Each task was divided into fluid (high rate of change in thought content) and non-fluid (little or no change in thought content) periods. After the task four questions were asked about the fluid and non-fluid period of the tasks. Subjects were instructed to answer honestly and accurately. The questions were concerned with the: (a) general content of thoughts, (b) rate of change of thoughts, (c) degree of off-task thoughts, and (d) required selecting an alternative on a five-point scale which went from "none or very little" to "very high or fast". Four equal time periods were considered to make the data comparable to earlier studies and to determine-time dependent or experience-dependent changes. Each period's means for both dependent measures are presented in Table 3. It can be seen that EM rate; (a) was similar for fluid and non-fluid thought under normal attention (NA) in each period, (b) was much higher for fluid than non-fluid thought under high attention (HA) in all periods with the difference declining with each successive period, and (c) was greater with HA than NA during any period of thought rate. Table 3 also shows that Percentage Change fluctuated about zero for NA while for HA the Percentage Change increased from Period 1 to 2 then successively decreased for Periods 3 and 4.

Questions 2, 3, and 4 indicated: (a) perceived rate of change in thought content greater during fluid than non-fluid thought intervals; (b) perceived degree of off-task thoughts greater under NA than HA, greater under non-fluid than fluid thought, and disproportionately greatest under the combination of normal attention and non-fluid thought; (c) and perceived need to suppress off-task thoughts unaffected by any experimental condition.

The results suggested that heightened attention increased EM rate regardless of thought content change rate. Also suggested was the limitation that an increased EM rate with an increase in change rate of thought content can occur only when attention level is high. Both these effects decreased with time spent on the task, perhaps due to a diminuation of the attention level of high attention Ss.

III. Daydreaming as a function of cueing and task difficulty.

The effects of demand characteristics and task difficulty upon the reported frequency of task-unrelated-thought intrusions (daydreaming) were investigated. As the literature in demand characteristics documents, a possible biasing effect exists whenever participants in an experiment become aware of the focus of the experiment in which they are participating; i.e., cues conveying the experimental hypothesis to an individual may cause him to alter his behavior to conform to the perceived hypothesis. Many studies of variables affecting daydreaming require a report of daydreaming frequency either for a prior time period or during an on-going task. The outcome and interpretations of such studies are open to question until the effect of asking about daydreams upon reported daydream frequency has been determined. Thus the primary purpose of this study was to determine the effect of cueing upon the reported frequency of daydreams.

The method employed to elicit daydreams is based on an attentional model which relates inner thought process (e.g., daydreaming) and external stimuli. The model assumes that, at any one time, attention is distributed between internal and external stimulation. By choosing a task which required minimal attention to external stimulation, it was expected that daydreams would be elicited. Furthermore, it was expected that by using two tasks which differed in their difficulty and demand for attention to external stimulation a further test of the model could be made by a comparison of reported daydream frequency.

Thirty-five male and 36 female college students were assigned to one of two control groups or an experimental group. There was an essentially equal proportion of males and females in each group. Table 4 portrays the overall design of this study. Two tasks differed in difficutly and hence amount of attention required for successful completion. Both tasks consisted of single X's visually presented every four seconds in one of four numbered windows located in a memory drum. Task 1 (the one-back task) was designed to require little constant attention to external stimuli and thus be conducive to daydreaming. In Task 1 the subject recalls and reports the window in which an X had appeared just prior to the X appearing to him at a particular time. Task 2 was designed to be more difficult and require more constant attention, and thereby to decrease daydreaming. It was similar to Task 1, except the subject recalls and reports the window in which the X had appeared two-back or three-back from the X appearing to him at that moment. For any one subject, Task 2 involved only two-back or three-back and not both. Each task took two minutes to complete. Each subject completed Task 1 twice followed by Task 2. Table 4 shows this task presentation order as well as the time at which each group was cued that task-unrelated-thought was being studied.

The notion that the subject's perception and reaction to demand characteristics will affect his performance led to the hypothesis that once the subject was cued that the experimenter was interested in the frequency of daydreaming during the task, reported daydreaming frequency would increase. The results of this experiment indicate that cueing produced not an increase but a decrease in daydreaming. The conclusion regarding a decrease due to cueing was strongly supported by the fact that in nearly every group following cueing a decrease occurred as well as from comparisons between independent groups having been cued and those not having been cued. The trend to reduce reported daydreams following cueing was especially evident in data set 2, suggesting that instances of daydreaming completely unrelated to the experimental setting were most often reduced or suppressed.

Significance to Bio-Medical Research and Program of the Institute:

The study of daydreaming is fundamentally a study of thought processes. Furthermore there has been little research in this area of thought processes especially across the entire adult age range. In order to understand fully the thought processes of man, the total spectrum of those processes need to be examined. In addition, one must know how this wide spectrum is affected by aging. Thus the study of daydreaming in adults, along with other variables such as differences in age, socio-economic status, attitudes, etc. may help us understand the fundamental processes which underlie all these behaviors.

Proposed Course of Project:

1. Intraindividual age changes in daydreaming is currently underway in the Baltimore Longitudinal Study;

2. Also underway is an investigation of the relationships of biological, social, and personality characteristics to daydreaming.

3. Data on females older than college age are currently being collected.

Honors and Awards:

Dr. Giambra was invited to lecture to the members of the student body at Miami University (February, 1974).

Publications: Giambra, L. M. Daydreaming across the life span: Late adolescent to senior citizen. Aging and Human Development (In Press).

Filler, M. S. and Giambra, Daydreaming as a function of cueing and task difficulty. Perceptual and Motor Skills, 1973, 37, 503-509.

Fiester, A. R., Mahrer, A. R., Giambra, L. M., and Ormiston, D. W. Shaping a clinical population: the dropout problem reconsidered. Community Mental Health Journal (In Press).

Table 1

Means For Each Age Group on Each Scale of the Imaginal Processes Inventory, Correlations of Each Scale With Age, and Partial Correlations of Each Scale With Age Holding Scale 1 Constant

Scale No.	Scale Description	Age Group 17-23	24-34	35-44	45-54	55-64	65-74	75-91	Correlation with age	Partial
28	Self-Revelation	26.9	21.0	21.5	23.1	20.2	20.2	21.7	-.32$	-.24$
4	Acceptance of Daydreaming	32.3	34.6	[illegible]	31.6	[illegible]	32.4	[illegible]	[illegible]	[illegible]
1	Daydreaming Frequency	23.0	23.6	[illegible]	21.1	21.4	19.6	[illegible]	[illegible]	-----
3	Absorption In Daydreaming	[illegible]	27.4	29.1	25.3	24.3	20.6	21.4	-.35$	-.17*
14	Mindwandering	24.7	26.8	25.4	23.5	[illegible]	24.1	[illegible]	[illegible]	[illegible]
22	Curiosity: Interpersonal	26.1	22.6	24.1	[illegible]	[illegible]	24.5	26.1	-.06	[illegible]
23	Curiosity: Impersonal-Mechanical	22.1	[illegible]	26.1	[illegible]	[illegible]	24.4	[illegible]	.14*	.11*
24	Boredom	21.9	19.4	17.0	[illegible]	17.1	16.0	16.0	[illegible]	-.26$
25	Mentation Rate	26.1	26.9	25.6	26.7	26.6	25.4	[illegible]	[illegible]	.04
26	Distractibility	26.1	[illegible]	25.3	[illegible]	21.2	19.7	[illegible]	-.37$	[illegible]
27	Need For External Stimulation	25.6	26.1	23.9	[illegible]	[illegible]	24.7	24.4	-.22$	[illegible]
10	Present Oriented Daydreams	23.3	24.8	25.2	24.7	23.4	23.1	24.1	[illegible]	-.04
11	Future In Daydreams	26.3	24.0	24.6	24.2	22.2	23.5	24.3	-.20$	-.11*
12	Past In Daydreams	24.2	18.7	23.4	20.6	22.4	23.8	25.5	-.02	.09
5	Positive Reactions in Daydreaming	25.3	21.7	26.1	21.3	19.8	19.0	19.5	-.31$	-.14*
6	Frightened Reaction to Daydreaming	17.0	11.2	10.4	14.0	11.6	12.6	9.8	-.29$	-.14*
7	Visual Imagery In Daydreaming	23.7	19.4	20.8	18.1	19.6	18.6	17.9	-.27$	-.10
8	Auditory Images In Daydreams	21.4	15.3	18.3	14.4	13.5	13.0	10.9	-.38$	-.24$
16	Hallucinatory-Vividness of Daydreams	11.5	7.0	9.6	9.2	6.2	7.2	7.1	-.25$	-.14*

Table 1 Cont'd.

Scale No.	Scale Description	Age Group 17-23	24-34	35-44	45-54	55-64	65-74	75-91	Correlation with age	Partial
9	Problem Solving Daydreams	23.3	21.7	25.8	21.3	17.9	20.4	19.3	-.19$	-.01
19	Sexual Daydreams	26.1	19.2	20.8	15.7	15.4	13.9	9.7	-.52$	-.38$
13	Bizarre-Improbable Daydreams	19.4	15.9	14.1	14.5	15.6	16.4	11.5	-.22$	-.14*
15	Achievement-Oriented Daydreams	18.9	15.3	14.1	11.6	11.9	9.3	14.6	-.29$	-.18$
18	Hostile Daydreams	16.6	9.4	10.9	7.5	9.1	7.7	4.7	-.44$	-.31$
20	Heroic Daydreams	16.0	9.3	7.8	7.2	7.7	6,9	6.9	-.40$	-.30$
17	Fear Of Failure Daydreams	13.0	7.8	8.6	9.2	9.0	7.3	5.4	-.33$	-.24$
21	Guilt Daydreams	11.3	6.2	6.6	5.3	6.1	5.5	4.3	-.31$	-.22$
2	Nightdreaming Frequency	23.0	14.3	15.9	11.9	15.7	14.8	11.4	-.35$	-.20#

* $p < .05$

# $p < .01$

\$ $p < .001$

Table 2

The Seven Factors After Varimax Rotation of the Intercorrelations of the 28 Scales of the Imaginal Processes Inventory and Age

Factor	Percent Variance	Name of Variable	Factor Loading
I	30.5	Guilt Daydreams	.87
		Fear of Failure Daydreams	.79
		Heroic Daydreams	.72
		Hostile Daydreams	.69
		Frightened Reactions to Daydreams	.59
		Achievement-Oriented Daydreams	.57
		Hallucinatory-Vividness of Daydreams	.51
		Absorption in Daydreaming	.38
		Age	-.36
II	9.3	Nightdreaming Frequency	.71
		Self Revelation	.53
		Mentation Rate	.49
		Past In Daydreams	.39
		Acceptance of Daydreaming	.32
		Age	-.38
III	7.2	Mindwandering	.85
		Boredom	.73
		Daydreaming Frequency	.71
		Distractibility	.66
		Sexual Daydreams	.61
		Absorption in Daydreams	.57
		Positive Reaction in Daydreams	.37
		Visual Imagery in Daydreams	.37
		Hostile Daydreams	.36
		Frightened Reaction to Daydreams	.32
		Age	-.47
IV	5.4	Present Oriented Daydreams	.86
		Problem Solving Daydreams	.40
		Bizarre-Improbable Daydreams	-.64

Table 2 Cont'd.

Factor	Percent Variance	Name of Variable	Factor Loading
V	5.0	Past In Daydreams	.55
		Age	.46
		Need For External Stimulation	-.80
VI	4.3	Future In Daydreams	.74
		Problem Solving Daydreams	.59
		Acceptance of Daydreaming	.56
		Curiosity: Interpersonal	.53
		Curiosity: Impersonal-Mechanical	.45
		Achievement-Oriented Daydreams	.43
VII	3.8	Auditory Imagery In Daydreams	.73
		Visual Imagery In Daydreams	.71
		Hallucinatory-Vividness of Daydreams	.58
		Positive Reaction In Daydreams	.47
		Curiosity: Impersonal-Mechanical	.46
		Absorption In Daydreaming	.43
		Past In Daydreams	.38
		Problem Solving Daydreams	.36
		Sexual Daydreams	.31

Table 3

Mean EM/Min. And Mean Percentage Change For Four Equal Time Periods

				Period			
Dependent Measure	Attention	Thought	Mean	1	2	3	4
EM/Min.	High	Fluid	82	94	80	80	73
		Nonfluid	61	61	53	68	62
	Normal	Fluid	49	50	54	48	43
		Nonfluid	48	48	50	52	41
Percentage Change	High	------	64	75	98	50	34
	Normal	------	2	1	7	-10	10

Table 4

Experimental Design--Sequency of Experiment and Information Obtained

Group	Period 1		Period 2		Period 3	
	Task Performed	Information Obtained	Task Performed	Information Obtained	Task Performed	Information Obtained
Control 1 (C1)	1	Task Difficulty	1	Task Difficulty	2	Task-unrelated-thought
Control 2 (C2)	1	Task Difficulty	1	Task-unrelated-thought	2	Task-unrelated-thought
Experimental (T)	1	Task-unrelated-thought	1	Task-unrelated-thought	2	Task-unrelated-thought

Table 5

Mean Frequency of Reported Task-Unrelated-Thought Intrusions for Period 1, 2, and 3

	Data Set 1 (All Reported)			Data Set 2 (Totally Unrelated To Experimental Situation)		
	Period			Period		
Male and Female	1	2	3	1	2	3
Group C1			1.46			0.79
Group C2		2.58	1.42		0.92	0.33
Group T	1.96	1.61	1.26	0.91	0.78	0.13
Male						
Group C1			1.17			0.67
Group C2		2.50	1.08		1.00	0.25
Groupt T	2.18	1.46	1.64	0.73	0.36	0.08
Female						
Group C1			1.75			0.92
Group C2		2.67	1.75		0.83	0.42
Group T	1.75	1.75	0.92	1.08	1.17	0.17

Serial No. HD-LSB-3
1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Physiological Psychology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Learned Modification of Visceral Function in Animals

Previous Serial Number: HD-AG 2 (8)

Principal Investigator: Bernard T. Engel, Ph.D. (55%)

Other Investigators: Sheldon H. Gottlieb, M.D. (90%)
Donald A. Kristt, M.D. (45%)

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	4.20
Professional:	1.90
Other:	2.30

Project Description:

Objectives:

A. Learned control of heart rate in monkeys.

To determine the physiological mechanisms associated with conditioned control of cardivascular functions in the rhesus monkey.

B. Depressor activity of the rat hypothalamus.

1. To locate and define vasodepressor areas in the rat diencephalon.
2. To explore the mechanism of this effect.
3. To develop a chronic, awake preparation in which such an effect can be elicited and continuously monitored.
4. To utilize this hypotensive effect to determine whether spontaneously hypertensive rats would engage in intracranial self-stimulation to obtain a transient lowering of blood pressure.

Methods Employed:

A. Learned control of heart rate in monkeys.

The subjects are adolescent rhesus monkeys. A catheter is placed in the aorta via the external iliac artery, and the animals are restrained in a primate chair contained in a sound-proofed booth. The arterial pressure pulse is detected by a transducer from which arterial pressure is measured directly, and the differentiated pressure pulse is used to give heart rate. The heart rate and blood pressure signals are interfaced with a Raytheon 704 computer which controls the experiment. Data from each session is then dumped on tape and analyzed by computer. Following initial shaping sessions, the animals are trained until they can reliably slow or speed their heart rate in response to a discriminative cue. The experimental period consists of 20 sessions of alternating speeding and slowing sessions followed by 40 slowing and speeding sessions during which autonomic blocking drugs are used to block either the sympathetic or parasympathetic system, and concludes with a final 20 sessions of control slowing and speeding sessions. The autonomic blocking drugs used are methyl-atropine bromide, 1 mg/kg and l-propranolol 1 mg/kg. Preliminary experiments showed that these drugs would effectively block the parasympathetic and sympathetic nervous system, respectively, and that no detectable drug effects remained by 24 hours after the last administration of the drug. The drugs were prepared fresh before use at a concentration of 1 mg/ml by dissolving the salts in normal saline and were given by intra-arterial injection at the rate of 2 mg/min.

Sessions were run from Monday through Friday. Four sessions were run each day. During the portion of the experiment when blocking drugs were used, either methyl-atropine or l-propranolol was given on Monday and Wednesday and both drugs were administered simultaneously to block both divisions of the autonomic nervous system on Fridays. Each day, therefore, consisted of a 20 minute equilibration during which the animal sat undisturbed in a closed booth. An 8 minute control heart rate and blood pressure was obtained and either methyl-atropine, l-propranolol or both drugs were then injected. On the intervening, non-drug days and equi-volume of saline was injected. When the heart rate had re-equilibrated, usually after 2-3 minutes, another 8 minute baseline was obtained. This was followed by a 35 minute training session. At the conclusion of the training session 2 additional 8 minute baseline sessions were obtained. An additional dose of the appropriate amount of drug or saline was then given and the sequence repeated.

In addition to measuring heart rate and blood pressure during conditioning, an attempt has been made to measure stroke volume and hence cardiac output during conditioning. Animals in which the aorta has been catheterized as already described have had electromagnetic flow probes placed around the root of their aortas. The flow probe signal is conditioned and is processed by the Raytheon 704 computer simultaneously with the heart rate and blood pressure signal. The signal conditioner produces a voltage proportional to the instantaneous beat to beat stroke volume and this voltage level may be used as operant feedback for conditioning stroke volume. The peri-aortic flow probes are wrapped in fine dacron mesh and have been implanted in pilot animals for up to 4 months with little aortic damage.

B. Depressor activity of the rat hypothalamus.

Forty-seven male Wistar rats were utilized: 13 were 6 to 11 mos. of age; the remaining animals were 5 mos. of age. All acute experiments were performed under pentobarbital anesthesia. An arterial catheter (either femoral or carotid) was placed, and the animal was prepared and fixed in a sterotaxic apparatus. Blood pressure (BP) and heart rate (HR) were continuously monitored throughout acute experiments. An electrode was placed unilaterally so that its exposed tip was in the region of the antero-lateral hypothalamus medial to median forebrain bundle. Exploration was first performed with monopolar electrodes since it seemed likely that vasodepressor effects might involve several structures. Stimulation consisted of trains of square-wave pulses which were produced by a Grass square wave generator via an isolated constant current unit.

For refinement of the localization and elucidation of the mechanism of action of the stimulation a concentric bipolar electrode was utilized. Two vasoactive drugs were employed to produce changes in BP. Isoproterenol and ephinephrine were used in doses of 1-4 μg/kg body weight.

All rats were decapitated and their brains fixed in 10% formalin for gross and/or microscopic evaluation of electrode localization.

Major Findings:

A. Learned control of heart rate in monkeys.

1. Drug Study

Four animals have completed the entire protocol and preliminary data has been analyzed for 3 animals. The ability of the animals to raise or lower their heart rates following blockade of the autonomic nervous system with the drug combinations in the protocol was consistent among animal's. When the parasympathetic nervous system is blocked an animals ability to slow is unimpaired. However, it is unable to consistently speed. When the sympathetic nervous system is blocked, speeding is unimpaired. However, the animal is unable to consistently slow. When both branches are blocked the animal consistently showed small increases in HR which may be artifactual due to the large number of shocks it received because of the difficulty in modulating heart rate to avoid the shock. Two of the animals showed consistent increases in blood pressure during sessions when either sympathetic or para-sympathetics were blocked. These animals had little change in blood pressure during non-drug control sessions. One consistent relationship which we had noted in earlier studies was that the correlation between heart rate and blood pressure is positive and high during control sessions. This relationship is still true when either the sympathetic or para-sympathetic system is blocked. When both systems are blocked, the correlation remains positive but is low.

These data suggest, therefore, that heart rate slowing in this system consists of a reduction in total sympathetic activity, and heart rate speeding is produced by vagal withdrawal.

2. Stroke volume and cardiac output.

One pilot animal had a flow probe in place for 4 months. This animal had been in a previous study and was unable to speed. Preliminary data suggest that this animal's cardiac output decreased while slowing, although stroke volume, *per se*, showed little change. We plan to measure stroke volume and cardiac output under control conditions and after blockade of the autonomic nervous system in all of the subjects of the heart rate experiment. One animal which was trained to slow and speed has had a flow probe successfully implanted and we are in the process of collecting data from this animal.

B. Depressor Activity of the Rat Hypothalamus

1. Monopolar electrodes:

A3.6, H7.7, L1.2 is a site where maximum monophasic depressor activity (SME) is produced. Generally, the effect is stable and reproducible with a fall in mean BP of 22 mm Hg (range 10-36 mm Hg; n = 32 stimulation). Occasionally pressures will fall 30 to 40 mm Hg., and sometimes a considerably smaller response is noted. HR, pulse pressure or respiration did not change during stimulation. Activity in the horizontal plane also was explored. Moving down past SME the response becomes biphasic. An analysis of stimulation parameters indicated that decreasing the current density produces a smaller effect on pressure, however, this effect is not linear. Halving the current intensity produced a decrement of 1/3; lowering the current 4/5 halved the effect.

Histologic examination of the brains showed the tip site to be bounded by lateral hypothalamic neurons, the fornix and the cerebral peduncle. This agrees well with expected electrode placement.

2. Bipolar Stimulation

In general bipolar stimulation permitted the use of much lower current densities.

For these experiments, variation in BP that can occur in the course of an acute lightly anesthetized preparation were exploited. Spontaneous variations in BP were used to explore vasodepressor activity at different BP levels. Mean baseline fluctuations were 5.1 mm Hg (n = 28). It was observed that the magnitude of the depressor response (DR) is an increasing function of pre-stimulatory BP (BP_o). For instance, BP_o = 100 mm Hg is associated with DR of 10 mm Hg, whereas when BP_o = 160 mm Hg, the post-stimulation BP falls 30 mm Hg. This relation also is seen at higher stimulation current levels.

It also was of interest to evaluate the same series of stimulations for the effect of variations in baseline BP during the three min. prior to BP_o, namely, baseline BP trend (BP_t). The data show that DR is an increasing function of BP_t. For instance at BP_t of -4 mm HG/min, DR = 15 mm Hg; at BP_t of = 4 mm Hg, DR = 30 mm Hg. Also, at the site selected for bipolar stimulation, an antecedent pressor phase of the response often was present. The pressor response generally appeared inversely related to BP_o. It also appeared to be related to DR in that the pressor response was maximal when DR was minimal.

Stimulation by itself, either monopolar or bipolar, did not produce changes in HR or rhythm. Yet, brief spontaneous episodes of hypotension during baseline periods were accompanied by a reflex tachycardia. Although changes in pulse pressure were noted at times, no consistent pattern was evident. Respiration rate also appears unaffected by stimulation.

It was desired to test further the notion that BP_o and BP_t were important determinants of DR by administering vasoactive agents. During the acute drug effect phasic responses were reliably obtained when stimulation current was raised above standard levels. Epinephrine (which raised BP) was associated with a depressor response, and isoproterenol (which lowered BP) with a pressor response. Unexpectedly, on several occasions substantial changes in HR and rhythm did occur with stimulation. They were noted in the period of the acute drug effect. Epinephrine plus stimulation could result in a striking bradycardia, e.g., 220 BPM with widening of pulse pressure. Isoproterenol, which by itself was not arrhythmiogenic, when combined with stimulation resulted in a pronounced bradycardia (HR 200 BPM) and a bigeminal rhythm in one case. Stimulation induced BP changes were absent in this latter instance.

3. Chronic awake preparation.

Four rats have been prepared with chronic intra-arterial catheters and permanent intra-cranial electrodes in the vasodepressor area. Electrical stimulation of this area in the awake rate results in a biphasic pressor-depressor effect, with a depressor component of between 15-30 mm Hg depending on the stimulation parameters that are employed. These BP changes are associated with a bradycardia of between 15-120 BPM below baseline HR, again depending on parameters of stimulation. The DR changes little with alteration in these stimulation parameters, but the pressor effect (PR) and HR changes are diminished by parameters that minimize spread of the stimulating current.

The sensitivity of PR and DR to BP_0 also has been seen in this chronic awake preparation. For instance, intra-arterial infusion of phenylephrine, which raises BP acutely, results in an increased DR and decreased PR.

4. Mechanism study.

Twenty-five rats were used to explore certain components of the cardiovascular system that may be related to the depressor response.

A possible role for the peripheral baroceptors was evaluated by stepwise deafferentiation of the two major baroceptor inputs to the CNS, viz. vagal afferents from the aortic baroceptors and sinus nerve afferents from the carotid sinus in a series of acute experiments. Preliminary analyses of these data suggest:

(a) The aortic baroceptors facilitate DR, since bilateral vagectomy reduces the DR by 50% while BP remains at the same level.

(b) The carotid baroceptors inhibit DR, since bilateral carotid occlusion results in at least a 50% increase in DR.

(c) The sensitivity of DR to changes in baseline BP is maintained in the face of 'total' baroceptor deafferentiation.

(d) Cardiac stroke volume is depressed by stimulation independently of the reduction in peripheral vascular resistance. This is based on the observation that following total deafferentiation, stimulation frequently results in a greater depression of systolic BP than diastolic BP, i.e., there is uncoupling of the BP responses to stimulation.

(e) Electrical stimulation of the vasodepressor area has effects similar to endogeneous spontaneous cardiovascular responses. This is based on the finding that rats in which the BP uncoupling response is noted show spontaneous variations in BP where systolic BP falls more than diastolic. Conversely, rats in which pulse pressure remained uneffected by stimulations exhibited stable pulse pressure during spontaneous (non-stimulation induced) BP changes.

The role of the chemo-receptors also was evaluated. Rats were stimulated during periods of hypoxia or hyperoxygenation before and after deafferentiation. Activation of the chemoreceptors did not result in any detectably unique influence on the DR either before or after deafferentiation.

Two chronic awake rats (as described above) were given 15 mg/Kg of atropine intra-arterially and then electrically stimulated. There was marked attenuation of the DR. HR did not change in one subject, but did increase in the second at this dosage. Stimulation of these animals on the day following atropinization resulted in the usual magnitude of the DR.

5. Behavioral study.

Four normotensive and one spontaneously hypertensive (SH) rats (Okamoto-Aoki) underwent behavioral shaping in a standard operant conditioning chamber. These animals were being 'shaped' to depress a manipulandum for electrical stimulation of the vasodepressor area to determine if such stimulation was reinforcing. The four normotensive rats bar pressed on several occasions for short periods, i.e., 10-50 presses/10 min. Following these bursts of activity the rat would close its eyes and appeared to be somnolent for 10-20 min. The SH rat could be enouraged to stay with its head above the manipulandum but would become somnolent before attempting to bar press.

Possible conclusions regarding these data include:

(a) Stimulation of this area maybe mildly reinforcing,

(b) The transient lowering of BP in SH rats is not more reinforcing than in normotensive SS,

(c) Such BP changes may not be aversive and may induce somnolence.

Significance to Biomedical Research and the Program of the Institute:

A. Learned Control of Heart rate in monkeys.

These studies will help us to understand the cardiovascular mechanisms of heart rate control in this preparation. It is hoped that this knowledge will enable us to develop more effective training techniques not only for monkeys but also humans, and that this knowledge will help in understanding neural-cardiovascular integration.

B. Depressor activity of the rat hypothalamus.

The work performed to date can provide a basis for further exploration of hypothalamic cardiovascular regulation. The rat appears to offer the advantage that the spectum of regulatory maneuvers are much more limited than those of higher mammals.

Proposed Course of Project.

A. Learned control of heart rate in monkeys.

We will continue to study the cardiovascular changes with conditioning. In addition, we now have the ability to supply feedback to an animal for stroke volume. We plan to attempt to directly condition stroke volume. If this is successful, it would have broad implications regarding the mechanisms underlying the regulation of the work of the heart.

B. None

Honors and Awards:

Dr. Kristt conducted a seminar at the Bourne Behavioral Research Laboratory, Cornell College of Medicine (August 1973).

Dr. Engel delivered a paper at the annual meeting of the Pavlovian Society (November 1973).

Dr. Engel conducted a seminar at the Walter Reed Army Institute of Research (March 1974).

Dr. Engel conducted a seminar at the Department of Psychiatry, Montefiore Hospital (April 1974).

Publications: Ainslie, G. W. and Engel, B. T. Alteration of classically conditioned heart rate by operant reinforcement in monkeys. J. Comp. Physiol. Psychol., In Press.

Engel, B. T. Comment on self-control of cardiac functioning: A promise as yet unfulfilled. Psychol. Bull., 1974, 81, 43.

Serial No. HD-LBS-4
1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Learning and Problem Solving Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1973 through June 30, 1974

Project Title: Problem Solving and Age

Previous Serial Number: HD-AG6 (c)

Principal Investigator: David Arenberg, Ph.D. (50%)
Leonard M. Giambra, Ph.D. (50%)

Other Investigators: Phillip Thorne

Cooperating Units: Baltimore City Hospitals
Miami University of Ohio
College of Notre Dame of Maryland

Man Years:

Total:	2.60
Professional:	1.00
Others:	1.60

Project Description:

Objectives: The general goals are to explore and identify reasoning processes in man, to determine in what ways these processes change with age, and to develop techniques for reducing age deficits in reasoning performance. In this project, reasoning is studied by using problem-solving procedures in which on-going solution behavior can be observed and quantified. Experiments are designed to answer such questions as: (1) Is effectiveness in acquiring relevant information affected by aging? (2) Is effectiveness in synthesizing available information affected by aging? (3) What kinds of solution strategies are used and in what ways are they related to age? (4) How does imposing a memory load affect solution strategies for young and old adults?

Methods Employed: Experiment I is a longitudinal study of logical problem solving. The display board for all problems includes nine lights on the periphery of a circle and a goal light in the center. The peripheral lights can be turned on by switches. A subject's task is to light the goal light in a prescribed way. For each problem, a disk is superimposed on the light display panel. On the disk are arrows connecting various lights. An arrow indicates that one of three logical relations (previously explained to the subject) exists between the lights that arrow connects, but the specific relation is not identified. The solution of each problem requires lighting the goal light by a sequence of inputs[a] in which only the switches for a specified set of three (of the nine) peripheral lights can be used. Time between inputs is controlled by the subject. In addition to correctness of the solution, performance is evaluated by determining whether an input (at the time it is made): (1) provides new information, (2) provides inferable information, or (3) is overtly redundant.

Experiment X is a study in which the primary variable is the designation label. Pairs of labels are: + and -, A and not A, A and B, * and ⊕, Died and Lived. It is generally assumed in the concept literature that such labels or feedback messages are irrelevant. This study was designed to test the effects of labels. Other independent variables are sex, concept rule, and the effect of experience (comparing first, second, and third problems). The concept rules included are conjunction, disjunction, conditional, and biconditional.

Experiment XI is a concept study in which each subject solves a large number of problems. The literature in concept identification is based almost entirely on mean effects of subsamples in which each subject solves one or a few problems. It has not been established that the variables which affect the mean performance of groups would affect an individual in the same way. When a subject solves one or a few problems, his approach to reasoning can be characterized as unstable, variable, transient, and highly subject to chance occurrences. Solving many problems is expected to result in a steady state of performance. In that steady state, strategies can be more readily elicited and the effects of various independent variables on changes in strategy and other performance measures can be studied.

Major Findings: Experiment I, a longitudinal study of logical problem solving, has been completed. The 300 men in the initial sample were between 24 and 87 years of age at the time their performance was first measured. Cross-sectional results showed decreases with age in proportion of men who successfully solved each problem, and increases in the number of uninformative (inferable or redundant) inputs (see Tables 1 and 2). The mean interval between first and second measures was 6.70 years. Longitudinal measures of change showed a mean decline in performance only for the group over seventy initially (see Tables 1 and 2). Much of the decline was attributable to increased redundant inputs despite the fact that memory demands were minimized and the entire sequence of inputs and results was alway available. Furthermore, for the group over seventy, six-year survival was correlated with successful problem solving in the first session. Of the 36 men who successfully solved Problem I, 6 (17%) died during the six-year interval; of the 13 men who were unable to solve that problem, 6 (46%) died during that interval. It should be noted that a comparison of vocabulary means of those same 49 men over seventy showed no difference between those who survived (X = 68.9) and those who died (X = 69.3). The age changes which were found in this study of problem solving together with the longitudinal changes emerging from the learning and memory studies are showing a consistent picture of age deficits, particularly for men over seventy, in tasks which require dealing with new information. This is in contrast with the longitudinal findings in the literature. Those studies have not shown age changes for intelligence test performance, which is based primarily on stored (old) information.

---

[a] The term "input" refers to the situation in which one or more lights are on and a subject indicates he wants the results of those lights. Those lights are then extinguished, and according to the logical relations of the arrows pointing away from those lights, the resultant lights are turned on.

Experiment X is a concept study of the effects of feedback messages, the labels used to designate which instances are examples of a concept and which are not. The effects of the labels depended upon the concept rule; i.e., an interaction was found for labels and rules (see Table 3). When the labels were positively directional (e.g., + for positive instances and - for negative instances) or neutral (e.g., * for positive instances and ⊕ for negative instances), concepts with the conjunctive rule (cj) were learned in fewer trials than concepts with the inclusive disjunctive rule (id). When the labels were linguistically negatively directional (e.g., Died for positive instances and Lived for negative instances), there was a small difference in the opposite direction; the disjunctive concepts were learned at least as efficiently as the conjunctive concepts. A ceiling effect (many measures at or near the maximum of 27 trials) was found for the conditional (cd) and biconditional (bc) rules. It is now apparent that additional trials are needed to learn these concepts and to determine the effects of labels on performance with these more difficult concepts. The effects of labels on concept learning are complex; but it is already clear that labels do affect results. Almost all of the findings in the literature are based upon a single set of labels; and the results may be specific to the set of labels used and not generalizable to other conditions.

Experiment XI is a concept study in which many concept problems are presented in multiple sessions. The first subject, a young man, has attempted to solve 88 problems, and it is clear that steady state performance can be attained. One old subject has attempted only a few problems thus far, and no conclusions can be drawn at this time.

Significance to Bio-Medical Research and the Program of the Institute: Reasoning is among the most prized behaviors of man and among the most elusive for experimental study. In this project, methods have been and will be developed to obtain quantifiable measures of step-by-step performance on reasoning problems. Some of these methods also provide patterns of responses which represent strategies in solving such problems.

Measures are obtained in current experiments to study changes in reasoning processes with age. These studies, in addition to identifying basic reasoning processes, should indicate how pervasive reasoning deficits are with age, whether education and cognitive activity mitigate such deficits, and what techniques could be used to minimize decline in reasoning.

Proposed Course of Project: The changes in reasoning performance found in Experiment I will be related to blood pressure and other physiologic measures and to some specific diagnostic categories such as cerebrovascular disease and coronary artery disease. Performance on a similar problem solving task (Experiment IV) for an independent subsample of the Baltimore Longitudinal Study also will be related to those physiologic measures and disease categories, and the consistency of the findings can be determined. Additional subjects will be added to Experiment XI to determine the consistency of the findings and eventually the effects of age, sex, education, and intelligence. Those subjects who have reached a steady state will be shifted to a different set of problems in which variables known to affect performance of relatively naive subjects will be manipulated (e.g., number of irrelevant dimensions, concept rules, criterion for learning, etc.). Leads from previous pilot research on concept preference (Experiment VIII) will be pursued.

Honors and Awards: Dr. Arenberg was
Dr. Arenberg was a discussant in a symposium entitled "Translations in Gerontology--From Lab to Life" at the American Psychological Association Meetings in Montreal (September, 1973).

Publications: Giambra, L. M. An interactive effect of the criterion in concept identification: conditional and biconditional rule difficulty. Journal of Experimental Psychology, 1973, 97, 310-316.

Arenberg, D. A longitudinal study of problem solving in adults. Journal of Gerontology, In Press.

Table 1. Problem 1.

Proportions Who Solved and Mean Effectiveness

(Experiment I)

	Under 40	40s	50s	60s	Over 70
First session (N=300)	45	79	80	47	49
- proportion solved	.96	.94	.91	.79	.73
Returned (N=224)[a]	34	65	65	34	26
- proportion solved 1st session	.94	.97	.94	.79	.81
- proportion solved 2nd session	.97	.98	.95	.85	.82
Did not return (N=73)	11	11	15	13	23
- died (N=23)[b]	2,0	1,0	4,1	2,1	6,6
- withdrew (N=45)[b]	9,0	8,1	8,2	8,1	6,2
- ill (N=5)[b]	0,0	1,0	0,0	0,1	3,0
Mean number of uninformative inputs					
- all first session (N=263)	5.12	7.35	6.88	8.46	8.50
- solved both (N=193)	32	62	59	24	16
- first session	5.28	6.71	7.07	6.96	6.62
- second session	4.00	3.90	5.39	5.08	11.37
- change	-1.28	-2.81	-1.68	-1.87	4.75

[a] Three additional subjects returned but data could not be obtained

[b] Entries separated by comma indicate number who solved, number who did not solve.

Table 2. Problem II

Proportions Who Solved and Mean Effectiveness (Experiment I)

	Under 40	40s	50s	60s	Over 70
First Session (N=272)	45	77	76	40	34
- proportion solved	.87	.92	.87	.68	.71
Returned					
- proportion solved 1st session	.88	.92	.90	.60	.70
- proportion solved 2nd session	.94	.95	.87	.71	.69
Mean Number of uninformative inputs					
- all first session (N=227)	7.36	10.35	10.65	11.00	14.00
- solved both (N=166)	30	59	50	17	10
- first session	8.07	8.93	9.86	11.24	12.90
- second session	4.67	7.92	6.88	8.18	19.90
- change	-3.40	-1.02	-2.98	-3.06	7.00

Table 3

Mean Trial of Last Error for All Combinations of Concept Rule and Label

Rule	Directionality of Label				
	Strong Positive		Weak Positive	Neutral	Strong Negative
	+, -	A, not-A	A, B	*, ⊕	Died, Lived
cj	16.8	14.0	17.8	16.5	20.8
id	23.0	23.1	25.5	25.8	19.8
cd	24.3	24.0	25.0	24.2	25.3
bc	25.8	24.8	26.3	25.0	25.0

Serial No. HD-LBS-5
1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Learning and Problem Solving Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Verbal Learning and Age

Previous Serial Number: HD-AG7 (c)

Principal Investigators: David Arenberg, Ph.D. (40%)
Elizabeth A. Robertson, Ph.D. (90%)

Other Investigators: Phillip Thorne (10%)
Robert Vestal, M.D.

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	3.60
Professional:	1.40
Others:	2.20

Project Description:

Objectives: General objectives are: (1) to identify conditions which affect age differences in acquisition and retention of verbal material in man to understand aging processes and psychological processes of memory and learning; and (2) to identify conditions which are particularly beneficial to learning by the old to develop methods for training old workers and for education of the elderly.

Method Employed: Experiment XV was an age study of free recall in which lists of words were presented under three different conditions: (1) visual only, (2) visual plus passive auditory augmentation (the experimenter read each word aloud as it was presented), and (3) visual plus active auditory augmentation (the subject said each word aloud as it was presented). In free recall, the task is to recall as many words as one can after one presentation of the list.

Experiment XVIII is an aging study of paired-associate learning with three input conditions. In the acquisition trials, subjects are instructed to do one of the following: (1) look at each item, (2) look at each item and listen to the experimenter read it aloud, or (3) read each item aloud. Performance is measured in subject-paced test trials in which no feedback is provided. Previous studies (Experiments XII, XIII, and XV using these input conditions have shown that: (1) measures of primary memory (short-term) are facilitated by the two conditions with auditory components, particularly the "say" condition (read aloud); and (2) measures of secondary memory (long-term) are impaired by the two auditory conditions, particularly the "say" condition. This study is designed to determine the effects on rote learning of hearing and saying items while they are presented visually and thereby test the generality of the auditory effects on secondary memory.

Experiment XXV is a study of short-term recognition memory using signal detection procedures. On the basis of recent empirical data using signal detection methods, a mathematical model has been developed which provides measures of acquisition and decay for individual subjects. In Experiment XXV, auditory and visual presentation is used because under some conditions the elderly benefit more than the young from auditory inputs in memory performance. Recent studies also have shown ear differences in perception which have been attributed to differences in function of the cerebral hemispheres. An exploration of the acquisition and decay parameters for the two ears for subjects of different ages may provide some information about relative age deficits for the two hemispheres. In these recognition memory tasks, a series of items (digits and letters) is presented followed by a cue item. The task is to indicate whether the cue item had been presented in the series, and a certainty rating is made for each response. The acquisition and decay parameters are obtained by plotting a signal detection measure of memory against time (presented position). The slope of the line provides a measure of decay, and the intercept estimates strength of acquisition at time zero (at input before decay begins).

Experiment XXVI is a new short-term memory study in which mixed lists of digits and letters are presented and both recall and recognition tasks are used. The digits and letters are presented sequentially at a one second rate on contiguous screens. At the end of each list the subject is cued to answer one of three questions: (1) when the task is to recall the item which appeared on one of the screens, a question mark appears on that screen; (2) when the task is position recognition, an item (a digit or letter) appears on one of the screens, and the subject indicates whether that item had appeared on that screen; and (3) when the task is list recognition, an item appears on an additional screen, and the subject indicates whether that item had appeared anywhere in the list. A certainty rating is made for each response to permit signal detection measures to be calculated. The three tasks provide a comparison of two kinds of recognition under conditions which induce a recall strategy. It is assumed that the additional information required to respond successfully in the position recognition task imposes greater retrieval demands than does the list recognition task. On that basis, it is hypothesized that age differences will be smaller for list recognition than for position recognition. It is also hypothesized that the recall task will result in large age differences due to the substantial search aspects of retrieval required.

Experiment XXVII is a new study of dichotic listening and aging. A dichotic pair consists of two different digits simultaneously presented one to each ear. Frequently when two or more dichotic pairs are presented at a rapid rate, subjects recall all the digits to one ear before recalling any digits to the other ear. Previous studies have shown that age differences are small or not found in the half message (all the digits to one ear) reported first, but reliable differences are found for the half message reported second. Those differences have been interpreted as evidence for faster decay of stored information (the delayed half message) with age or for greater interference due to responding with the first half message. The simultaneity of pairs was not maximal in those studies. Simultaneity of pairs in the current study was achieved by computer timing using the GRC Raytheon 706 general-purpose-computer. Twenty-eight single pairs, 35 double pairs, and 35 triple pairs are presented to each subject. In addition to the dichotic messages, mixed messages in which each digit is presented to both ears are also used. This provides comparisons of ear-order responding under mixed and dichotic conditions. It has long been thought that ear-order responding in dichotic listening means that the ears operate as separate information channels with a store for each channel. Recent studies have shown that ear-order responding can occur even when both digits of a pair are presented to both ears, i.e., mixed. Those findings challenge the channel interpretation, because the procedure does not permit the ears to operate as separate channels. Ear-order responding with mixed pairs has been interpreted to mean that: (1) the auditory system is a sequential information processor, and (2) that the two ears do not serve as separate information channels contributing to ear-order responding. The second point has not been put to test, for it requires a comparison of frequency of ear-order responding under dichotic and mixed conditions. Such a comparison has not been reported, but will be made for several message lengths in this study. It will also be possible to compare the two conditions for young and old groups.

Experiment XXVIII is a new study of the effects of alcohol and aging on several memory tasks and decision tasks. The study of ethanol metabolism in the Clinical Physiology Branch provides an excellent opportunity to relate individual fasting blood-alcohol levels and rates of change to performance measured at fixed intervals prior to, during, and after infusion. The measures include immediate free recall, delayed free recall, delayed recognition, memory span, dichotic listening, simple reaction time, and choice reaction time at four levels of complexity. Three adult age levels are included. The measures were selected to identify cognitive processes which are affected by alcohol and processes which are not.

Major Findings: Experiment XV is a free recall study with three presentation conditions and two age groups (young and old men). A similar study (Experiment XVa) by Dr. Robertson has shown that, for both visual and auditory presentation, intrusion errors (words from a previous list recalled inappropriately for later lists) were age related. At each of the four conditions of that study, the old committed more intrusion errors than the middle-aged who committed more intrusion errors than the young. The results of Experiment XV corroborate those findings. At each of the three conditions the old group committed more intrusion errors than the young (see Table 1). Some of these errors were words from previous lists which had not been recalled at that time and the old committed more of those errors than the young. This finding, too, was similar to Experiment XVa. In order for those words to be recalled later, they must have been in store earlier but not accessible then. This is further evidence of an age deficit in retrieval.

Experiment XVIII is a paired-associate learning study. On the basis of previous research in this laboratory, it was hypothesized that saying items aloud or hearing them spoken as they are seen would be detrimental to learning, particularly for the old. The results did not support these hypotheses (see Table 2). Although the age effect was strong as in all previous verbal learning studies, the condition effect was small. For both age groups, the "listen" condition was somewhat more conducive to learning than the "look" condition, and the "say" condition was intermediate. Clearly the addition of an auditory cue to visually displayed material is not detrimental to secondary memory in all procedures, and a satisfactory explanation for the difference has not yet been found. Rate of input and output rate can be ruled out, and the paired-associate procedure used was much like the free recall procedure of the earlier study in that input and output aspects of the task were separated. One major difference was in the repetitive aspect of paired-associate learning. The same items are presented many times in paired-associate studies but only once in the free recall procedure. It may be that auditory augmentation is conducive to learning when the same material is presented over multiple trials.

Experiment XXV is the signal detection study of short-term recognition for visual and auditory stimuli. The visual procedure has been developed and is ready. The auditory tapes are in preparation, and data collection will begin in the very near future.

Experiment XXVI is a new study of recall and recognition of mixed lists of letters and digits. Few subjects have been studied thus far, but the data suggest that the greater retrieval demands of position recognition than list recognition is resulting in better memory performance for the latter condition, as predicted.

Experiment XXVII is a new study of dichotic listening. Preliminary data indicate that many men over sixty were rarely able to report both digits of a single dichotic pair. Young men almost always reported both digits (see Table 3). Although the results of aging studies of dichotic listening in the literature have been interpreted as strong evidence for a memory deficit with age, it is clear that the failure to identify both digits of a single pair is not attributable to memory. The same men who cannot report both digits can recall six or more digits in the correct order when they are presented sequentially. These data indicate that at least some part of the age differences in dichotic listening reflects a perceptual or attentional deficit rather than a memory deficit.

Experiment XXVIII is the new study of the effects of infused ethanol on cognitive performance. Although only a few young and a few old subjects have been studied, one dramatic effect is already clear. Delayed free recall has dropped to zero at peak blood alcohol levels for virtually everyone. Digit span (the maximum number of digits recalled correctly in order) seems to be little affected even at the peak alcohol levels.

Significance to Bio-Medical Research and the Program of the Institute: Learning is more central to experimental psychology than any other behavior and some of the most striking and consistently reported behavioral age differences in the gerontological literature have been found in verbal learning performance. The experiments in this project are designed to identify basic mechanisms of learning and retention and to measure changes in these functions that occur with age.

Aging, viewed as a natural experiment in which many changes occur, permits one to identify a variety of processes which may be important underlying mechanisms of learning. In addition, knowledge about experimental variables which affect age differences will be valuable in developing techniques for optimizing learning of the older person.

Proposed Course of Project: A study of dichotic listening using precueing and postcueing has been planned to explore the role of attention in the difficulty in responding with both digits by the old. Furthermore, by offsetting the digits in a pair, it may be possible to estimate the time required by an individual to shift his attention from one information channel to another. With the exquisite control of the stimulus timing provided by the Raytheon 706 general-purpose computer, it is now possible to increment the interstimulus interval between the presentation of two digits in callibrated steps as small as one millisecond. The effect of auditory augmentation on learning repeated material will be studied using free recall of words. The longitudinal data on learning and memory will be related to blood pressure and other physiologic measures.

Honors and Awards:

Dr. Robertson was invited to speak at the annual meeting of the Maryland Retired Teachers Association (October, 1973).

Dr. Arenberg was invited to lecture in Seattle at the summer course on psychological and social aspects of aging sponsored by NICHD (June-July, 1974).

Publications:

Arenberg, D., and Robertson, E. A. The older individual as a learner. In W. D. Mason, and S. Grabowski (Eds.), Education for Aging, Washington: Adult Education Association, In Press.

Table 1. Number of Total Intrusion Errors (Experiment XV)

	Condition			Total
	Say	Listen	Look	
Old (N = 42)	62	74	78	214
Young (N = 42)	28	30	31	89

Table 2. Mean Errors (Experiment XVIII)

	Condition		
	Say	Listen	Look
Old	42.1 (N = 20)	38.5 (N = 20)	48.6 (N = 20)
Young	18.9 (N = 31)	10.9 (N = 31)	19.1 (N = 32)

Table 3. Mean Number of Dichotic Pairs (Experiment XXVII)

	Both correct	Only one correct	Neither correct
Old (N = 32)	15.2	11.3	1.5
Young (N = 19)	26.3	1.7	0.0

Serial No. HD-LBS-6
1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Learning and Problem Solving Section
4. Baltimore, Maryland 21224

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Perceptual Retention and Age

Previous Serial Number: HD-AG8 (c)

Principal Investigators:	David Arenberg, Ph.D.	(10%)
	Elizabeth A. Robertson, Ph.D.	(10%)

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	.30
Professional:	.20
Others:	.10

Project Description:

Objectives: The general objectives are to investigate the effects of interference in perceptual retention and in perception: (1) to determine whether aging results in increased susceptibility to interference; (2) to explore conditions which affect age differences in interference; and (3) to hypothesize and develop procedures for testing mechanisms which may account for the empirical findings.

Methods employed: In Experiment V, as in all previous experiments in this project, the basic retention paradigm consisted of three sets of stimulus presentations: initial, interpolated, and final. Each stimulus presentation was a light or sound presented for a specific period of time and the subject was required to identify different durations by the labels that had been provided. The periods that the light could be on in the initial and final sets were 0.20, 0.35, 0.62, and 1.10 sec. The four periods were verbally labeled "one," "two," "three," and "four" in order, and eight practice judgments were confirmed or corrected. This was followed by the primary task of the initial set which was to identify 32 periods during which no feedback about the correctness of judgment was provided. Performance was measured by the number of incorrectly identified stimuli. The periods for the interpolated set were 1.10, 1.61, 2.14, and 2.68 sec. These were labeled, practice with feedback was provided, and then interpolated stimuli were presented without feedback. The final set consisted of 32 presentations of the periods used in the initial set, but no practice was provided. The primary data consisted of the change in performance (in number of incorrectly identified stimuli) from the initial to the final sets. The conditions of the experiment were determined by the procedures for the interpolated set. Previous experiments (I, II, & III) had shown consistently that errors increased more for old than for young men when the interpolated set consisted of visually presented stimuli labeled "one", "two", "three", and "four". In a more recent study (Experiment IV), it was shown that at least part of the age difference in interference was due to confusion introduced by the use of the same labels for the interpolated and the other sets of stimuli. When the labels for the interpolated set were "four", "five", "six", and "seven", the age difference in interference was substantially reduced. In Experiment V, the labels used for the interpolated set and the modality (visual or auditory) of those stimuli were included as independent variables.

Major Findings: Experiment V, in which modality and confusability due to labels were independent variables, was completed. The results were similar to the preliminary results reported last year. Just as in previous experiments (II, III, & IV), every interpolated condition produced an interference effect; i.e., errors of time judgment increased following the interpolated judgments. Furthermore, in all four combinations of modality and labels, interference effects (as measured by the mean increase in errors after interpolated judgments) were greater for the old than for the young. The largest age difference in interference effects occurred for the interpolated condition in which the stimuli were presented auditorily and the labels were confusing (the same labels as the short set of stimuli).

The results of these studies can be understood best by inspecting the summary data for all conditions in all the experiments in which the initial and final procedures were the same (see Table 1). Those interpolated conditions which were identical were grouped together to demonstrate the consistency of the results from several studies. The three primary findings were:

1. All interpolated conditions consistently (for 14 independent experimental groups) produced interference for all young and old groups, but interference was not found for a young or an old group in the control condition in which interpolated stimuli were not presented (the time was filled with non-interfering activities).

2. All old groups but one showed more interference than all seven young groups.

3. The variables of modality and label had no effect for the young; the mean interference measures were remarkably similar among the seven young groups with interpolated stimuli. For the old, however, both modality and label affected interference. At each label condition, auditory interpolation resulted in more interference than every group with visual interpolation; and at each modality condition, the confusing labels (1, 2, 3, 4) were consistently more interfering than the non-confusing labels (4, 5, 6, 7).

The large interference effect of auditory stimuli for the old groups was surprising because it contradicts the principle of similarity based on an extensive (non-gerontological) literature; the less similar the interpolated stimuli are to the initial and final stimuli, the smaller is the interference effect. A potential explanation of the larger rather than the smaller interference effect of auditory stimuli on judgments of visual stimuli is based upon the subjective difference in perceived time for auditory and visual periods. A consistent finding in the literature is that auditory periods are subjectively longer than objectively equivalent visual periods. It has also been shown that old as well as young subjects perceive auditory periods as longer than visual periods of the same length, and the effect was the same for old and young groups. If increasing the interpolated periods would result in increased interference for the old, then the interpolated auditory periods, which are functionally longer than (but objectively equal to) the interpolated visual periods we have used, would result in greater interference; and that is what we have found.

Significance to Bio-Medical Research and the Program of the Institute: The general idea that a person becomes more susceptible to interference as he grows older is well entrenched in gerontological thinking and is often used to "explain" age differences in performance. The evidence for this idea, however, is sparse and not consistent. It is the purpose of this project to explore the generality of the age-interference hypothesis for non-verbal memory and perception. It is important, both for theoretical and applied reasons, to identify those conditions which are especially interfering for the old.

Proposed Course of Project: Studies have been planned and data collection will be initiated to pursue the hypothesis (based on the recently completed study) that long auditory periods interfere more with memory of short visual periods than do long visual periods because the auditory periods are subjectively even longer than the long visual periods. An experiment designed last year (Experiment VI) to study speed of information processing has been modified and will be initiated. Other experiments planned in this project will explore age effects of more basic perceptual interference phenomena such as masking and disinhibition.

Honors and Awards: None

Publications: None

TABLE I

MEAN ERRORS FOR ALL COMPARABLE EXPERIMENTS

Interpolated Conditions			Old				Young			
Modality	Labels	Exp't	N	Initial	Final	Change	N	Initial	Final	Change
Visual	1,2,3,4	II	35	7.83	14.29	6.46	35	7.43	10.77	3.34
Visual	1,2,3,4	III	50	9.04	16.23	7.24	50	8.46	12.88	4.42
Visual	1,2,3,4	V	16	9.62	15.18	5.56	20	6.45	11.05	4.60
Visual	4,5,6,7	IV	72	9.47	12.29	2.82	78	7.60	10.94	3.34
Visual	4,5,6,7	V	16	7.25	12.43	5.18	20	6.47	10.33	3.86
Auditory	1,2,3,4	V	16	6.68	14.81	8.13	20	8.15	11.75	3.60
Auditory	4,5,6,7	V	16	6.81	12.18	5.37	20	7.85	11.35	3.50
No Interpolated Stim. (Control)			51	9.33	9.80	0.47	51	7.02	6.43	-0.59

Serial No. HD-LBS-7
1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Physiological Psychology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Reaction Time and Age

Previous Serial Number: HD-AG-14(8)

Principal Investigator: Bernard T. Engel, Ph.D. (5%)

Other Investigators: Phillip R. Thorne (5%)

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	.50
Professional:	.10
Other:	.40

Project Description:

Objectives: The purpose of this project is to study the effect of delay time on human reaction time, and to evaluate the function of age on this effect.

Method Employed: In the reaction time procedure used in this project, a subject is presented with an auditory signal (a tone to which he is supposed to depress a thumb switch as quickly as possible). The time between the onset of the tone and the switch press is the measure of his reaction time. Since the purpose of this project is to study the effect of time delay on reaction time, two time periods preceding each tone are varied systematically: (1) The time immediately preceding the onset of the tone--the foreperiod--is signalled by a light which goes on before the tone, and which remains on until the tone sounds: (2) The time between the end of one trial and the onset of the light of the next trial--the intertrial interval. By means of an appropriate statistical design, the effects of each of these time periods on reaction time, and the effect of the interaction between them on reaction time can be systematically studied.

Major Findings: Two projects were carried out. In the first study the intertrial interval (ITI) and the foreperiod (FP) each were varied systematically: All of the combinations of 3 sec, 6 sec and 12 sec were tested. Subjects in this study were 20 young men (age 20-30) and 20 older men (age 50-60). The results were that reaction time (RT) decreased monotonically as a function of FP, and increased monotonically as a function of ITI. The older group was consistently slower than the younger group, however, there were no interactions between age and FP or age and ITI. An analysis of the interaction between FP and ITI revealed a monotonic increase in RT as a function of the ratio of FP to ITI.

The second study was designed to evaluate the relationship between RT and the ratio of FP to ITI. In this study the trial length was held constant at either 12 sec or 24 sec and the ratio of FP to ITI was varied systematically. The following FP:ITI ratios were employed: 5:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:5. Once again it was seen that RT was a monotonic, increasing function of FP/ITI.

Significance: The primary purpose of this project was to investigate the relationship of timing mechanisms to age. The results clearly showed that the RT model did not discriminate among age groups. Although the older men were slower than the younger men, the RT process was similar for both age groups. The results of the interaction between FP and ITI are significant to the understanding of timing mechanisms in man, and these data will be reported.

Proposed Course: This project will be discontinued.

Honors and Awards: None

Publications: None

Serial No. HD-LBS-8
1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Physiological Psychology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Learned Modification of Visceral Function in Man

Previous Serial Number: HD-AG 16 (8)

Principal Investigator: Bernard T. Engel, Ph.D. (40%)

Other Investigators: Donald A. Kristt, M.D. (45%)
Marvin M. Schuster, M.D. (5%)

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	2.20
Professional:	.90
Other:	1.30

Project Description:

Objectives:

A. Learned control of blood pressure.
   1. To determine whether patients with essential hypertension can be taught to control their systolic blood pressure (SBP) through operant conditioning techniques.

   2. To determine whether patients so trained can achieve a significant and sustained lowering of their abnormally elevated blood pressure.

B. Learned control of anal sphincter function.
   1. To determine whether patients with severe, intractable fecal incontinence (continuous daily soiling) can be trained to control anal sphincter responses.

   2. To determine whether responses of the internal as well as the external anal sphincter can be brought under voluntary control.

   3. To determine whether such control, once learned will lead to clinically significant improvements in lower bowel function.

Methods Employed:

A. Learned control of blood pressure.

Volunteer patients are recruited from the Baltimore City Hospitals (BCH) Hypertension Clinic. During the study patients are hospitalized for three weeks on the research ward of BCH. Patients are trained in the laboratory to raise and lower median SBP. During a typical experimental session the patient lies semireclined in a hospital bed. Arranged in front of him are a vertical display of three lights. The top light is green, the middle light is yellow and the bottom light is red. In addition, there is a digital score meter that provides the patient with a cumulative record of his success during a trial. A standard blood pressure cuff, covering a small crystal microphone, is applied over the ante-cubital fossa. The microphone detects the presence of Korotkoff's sounds (KS).

Each session is divided into three phases. Phase 1 is a determination of baseline SBP. Before going on to phase 2, the patient's blood pressure has to become stable over at least five trials. Each trial is 25 heart beats in duration. Following the 25 beat trial the BP cuff is deflated and the patient is permitted to rest for 15 sec. before the next trial.

Phase 2 consists of 3 batteries of 10 training trials each separated by a short pause. During this phase the patient tries to control his blood pressure. Phase 3 is a post-training SBP baseline of 5 trials.

If the patient is being trained to raise his blood pressure, the green light is turned on and remains lit throughout the training trial. Lights are off during baseline phases, and inter-trial rest periods. When the patient raises his SBP (i.e., produces more (KS) at a fixed cuff pressure in a given trial), the yellow light goes on and remains on as long as KS are produced. If the patient's SBP fell below cuff pressure for a given heart beat and no KS was produced, then the yellow light would go off. Each heart beat accompanied by a KS during "raising" training increments the cumulative score meter by 2. Each patient is instructed to keep the yellow light on as much as possible and to accumulate as high a score as possible.

If the patient is being trained to lower his SBP, reinforcement (the yellow light) is provided when production of KS is suppressed. In this case the score meter reflects the number of heart beats not accompanied by KS.

Cuff pressure was adjusted on a trial to trial basis so that 25-75% of Korotkoff sounds were either produced or suppressed each trial; i.e., cuff pressure was adjusted to match median SBP for that trial.

After the patient has been trained separately to raise and to lower his SBP, he is further tested under differential training conditions. During the training phases of these sessions the patient is required to alternately raise and lower his SBP over blocks of 10-15 consecutive trials. The onset of each training condition is signaled by the appropriate cue lights, either green or red.

On discharge from the hospital the patient is instructed to practice an exercise using a self-applied blood pressure cuff. The exercise consists of inflating the cuff to the level of SBP and then attempting to make the KS disappear which means that SBP has fallen. The patient then determines the new lower level of SBP. The patient keeps a daily record of the original and final SBP. In the 3 mos. follow-up period in which the patient is practicing this exercise he is also recording systolic and diastolic BP daily.

In addition two formal follow-up sessions are scheduled in the laboratory: one at one month and one at 3 months post-hospitalization. In these sessions the patient is asked to alternately raise and lower SBP.

B. Learned control of anal sphincter function.

Sphincteric activity is monitored by means of pressure recordings from a triple balloon system. One balloon is placed in the rectum, a second balloon is placed at the internal sphincter and the third balloon is placed at the external sphincter. In normal subjects distension of the rectum elicits reflex relaxation of the internal sphincter, and simultaneous contraction of the external sphincter.

Each patient in this study was evaluated in three ways: (1) his clinical history was taken; (2) rectal examination was conducted to determine clinically the degree of sphincteric tone; (3) the rectal distension procedure described above was employed: (a) to determine which sphincter was malfunctioning, and (b) to determine the threshold of response of the sphincteric reflex. Thresholds were determined by systematically inflating the rectal balloon with a known volume of air and simultaneously observing the sphincteric responses.

Once the patient's threshold response was determined, a series of baseline recordings was made to provide an objective basis for measuring change. After the baseline measurements were made, the patient was permitted to observe the tracings from the pressure manometer, and he was instructed to "make the responses look like normal ones." The patient had received instructions earlier on the nature of normal sphincteric activity. Depending upon the particular abnormality the patient manifested, he would be instructed to try to control either sphincter or the synchrony between the sphincters.

Patients were seen in one to three training sessions separated by periods ranging from two to six weeks. Improvement was measured in terms of change in sphincteric activity in the laboratory, in terms of the physicians judgement of the change in anal sphincteric tone and in terms of the patient's report of his ability to remain continent.

Major Findings:

A. Learned Control of blood pressure.

Five patients were seen. Their ages range from 46 to 70 years. They all have been diagnosed as having essential hypertension and the duration of their disease exceeds 10 years in all cases. The four patients taking anti-hypertensive medication were under good pharmacologic control prior to entering the study. However, pre-treatment BP levels showed mild to severe elevations in BP. During the period of their training, while they were hospitalized, medication schedules were adjusted to obviate the common hypotensive side effects resulting from hospitalization of active, treated hypertensives. Following training the four patients receiving anti-hypertensive medication could be controlled on these lower dosage schedules.

The first stage of training consisted of 11 to 15 raising sessions. All patients were able to perform successfully. The mean elevation over baseline SBP for the last four of these sessions was 16 mm Hg for the five patients. The second stage consisted of 11 to 14 lowering sessions. The mean lowering of SBP for the last four of these sessions for the five patients was 12 mm Hg, and all patients were able to perform this task successfully. During the last stage of training in which patients were asked to alternate lowering with raising, in the order lower - raise - lower, the mean changes in SBP were (L_1) - 7, (R_1) + 12 (L_2) - 12. Again all patients performed successfully. Occasional sessions in several patients followed the order R-L-R with equivalent results.

Heart rate was monitored for all sessions. An analyses of the last four sessions of each stage of training showed considerable variation from patient to patient. The average group changes were slight. For instance, mean HR (beats/min.) for the three trials blocks in the last four alternate sessions was (L_1) 57.6 (R_1) 57.7 (L_2) 57.0. Respiration was monitored in several alternate sessions in four patients. Preliminary evaluation shows no substantial change in rate between lowering and raising trial blocks.

Systolic ejection times (performed according to method Weisler) were studied in two patients. No differences were evident between raising and lowering.

Four patients have completed the 3 month follow-up. These data show decreases in SBP post training of from 5-37 mm Hg based on 112-280 home determination/patient in the follow-up period. The data from the lowering practice show that patients can lower SBP during this exercise from 10 to 20 mm Hg. These are mean changes based on 5-30 readings/day for 4-10 weeks. This has been confirmed by the principal experimenter in 3 patients and verified in one of these patients by the chief of the BCH hypertension clinic as well.

In the short follow-up period available no change in EKG, chest X-ray or eye grounds were evident as compared to pre-training status.

The performance in the follow-up alternate session in the laboratory was: (a) at 1 mo. - (L_1) - 7.4, (R_1) 11.8, (L_2) - 9.0, (b) at 3 mos. - (L_1) - 11.5, (R_1) 12, (L_2) - 12.5. Data on only 4 patients are available for the 3 mos. sessions.

B. Learned control of anal sphincter function.

Each of the six patients who were seen last year continue to remain continent on follow-up. Three additional patients were seen by a Fellow in gastroenterology this year. He was able to train each patient to become continent within three training sessions or fewer, and all of the patients remain continent. Two of the patients are especially interesting. One was a woman who suffered from a prolapsed rectum as well as from incontinence. Within the year since she was last studied she has not had another prolapse. The second patient had an aterio-venous malformation at the third lumbar level: Physical signs included foot drop, urinary incontinence and fecal incontinence. Following corrective surgery (done in 1969) the foot drop and urinary incontinence was corrected, however, the fecal incontinence remained. Following operant training the patient is no longer incontinent. These findings coupled with our results from last year show the training technique can be learned readily and that it can be used to treat a number of related rectal motor disorders. In view of the fact that there are no effective alternative therapies, it would appear that operant conditioning is the treatment of choice for this disorder.

Significance: This project is designed to increase our understanding of learned control of autonomic responses and the application of this knowledge to the control of various illnesses. These data add further credence to the conclusion that responses mediated by the autonomic nervous system can be brought under voluntary control, and they show that such control holds out the possibility of clinically useful applications.

Proposed Future Course:

A. Learned control of blood pressure.

The results of this study open up a number of significant new research possibilities.

(1) Further work will be done with patients with essential hypertension, perhaps coupling operant conditioning with relaxation training.

(2) Patients with angina pectoris will be trained to control systolic blood pressure and heart rate simultaneously. Such training should permit the patients to significantly attenuate the pain associated with this disorder.

B. Learned control of anal sphincter function.

Patients with irritable bowel syndrome will be studied using a method similar to the one described above for training patients to control fecal incontinence. The goal of this research will be to train these patients to relax rectal smooth muscle.

Honors and Awards: Dr. Engel conducted medical grand rounds, Baylor College of Medicine (August 1973).

Dr. Engel conducted Psychiatry residents'staff conference. Walter Reed Army Hospital (August 1973).

Dr. Engel lectured in a course on Human Biology. University of Missouri School of Medicine (February 1974).

Dr. Engel conducted Medical Staff conference. Holy Cross Hospital, Bethesda (March 1974).

Dr. Engel participated in a workshop on Behavior Modification. American Psychosomatic Society (March 1974).

Dr. Engel conducted Psychiatry grand rounds. University of Wisconsin School of Medicine (April 1974).

Dr. Engel participated in a Symposium of the American College of Cardiology (May 1974).

Dr. Engel lectured in a course Psychosomatic and Psychiatric principles in medicine. University of Maryland School of Medicine (May 1974).

Dr. Engel participated in a Symposium of the Society for Psychotherapy Research (June 1974).

Publications: Engel, B. T., Nikoomanesh, P. and Schuster, M.M. Operant conditioning of recto-sphincteric responses in the treatment of fecal incontinence. New England Journal of Medicine, 1974, 290, 646-649.

Engel, B. T. Learned, voluntary control of cardiac function. Cardiology Today, In Press.

Serial No. HD-LBS-9
1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Learning and Problem Solving Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Operant performance, memory and aging

Previous Serial Number: HD-AG-25

Principal Investigator: Charles L. Goodrick, Ph.D. (30%)

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	.60
Professional:	.30
Others	.30

Project Description:

Objectives: Research on both human and animal subjects has shown that short-term memory deficits and motor performance deficits are major problems of aged individuals. An interpretation of maze learning differences between young and aged rats is that these differences are due to short-term memory deficits of the old animals. The general objectives of one phase of this project are: (1) to analyze complex maze learning of young and aged animals; and (2) to determine variables which may act to enhance or retard maze learning ability. The objectives of the other phase of this project are: (1) to study age differences in motor performance during operant responding; and (2) to develop operant techniques which improve the retention of learned responses in aged animals.

Methods Employed: Operant conditioning performance and retention studies have used 2-bar test boxes in which hungry animals are trained to press one bar to obtain a food reward while the alternate bar remains neutral. By increasing the complexity of the task (using two bars rather than one), it is possible to make a finer analysis of performance and the retention process. We are studying performance and retention as a function of reward schedule, and we are particularly interested in the partial reinforcement effect. The retention of partially rewarded responses is vastly greater than responses continuously rewarded; and analysis of this phenomenon will provide information regarding the general retention process. A complex 14-unit multiple-T maze also is utilized. This maze has been shown to be a highly reliable test of memory, and it has been used in many major studies of aging and learning. Additional mazes of 6-units are being developed to study mastery of consecutive problems by young and aged rats. These mazes will be used to analyze aging effects in short-term and long-term memory and to determine aging effects in proactive and retroactive inhibition.

Major findings:

1. Previous research found that aged rats learned complex problems more slowly than mature-young rats and that this learning deficit was related to distribution of practice during learning. A major purpose of the present experiment was to determine possible differential age effects with respect to facilitation of complex maze learning by the use of preliminary forced correct response training. A second purpose was to study the effect of distribution of practice upon reduction of time and error scores after forced correct response training. The subjects were mature-young (5 month old) and aged (25 month old) Wistar rats. Data were obtained for twenty-eight mature-young rats and twenty-eight aged rats. Over a three week period, all subjects were reduced to 75% of ad libitum body weight, gentled, and given pre-training trials in a straight runway to obtain one milliliter of condensed milk - sucrose (10%) reward solution.

Following pre-training, all rats were given 20 trials of training (4 per day) in a 14-unit multiple T-maze. During this stage of the experiment all 14 wrong turns were blocked, so that the animals ran the correct path to food reward. Following this training procedure, all animals received 16 test trials with the barriers removed. During test trials one-half of each age group was tested with one trial per day (distributed test trials), while the remainder were tested with four trials per day (massed test trials). Repeated measures analyses of variance were computed for time scores during training trials, and for both time scores and error scores during test trials. The experimental design for training trials was 2 × 5 × 4, age × series × trials and the experimental designs for test trials were 2 × 2 × 5 × 4, age × practice condition × series × trials. During training trials, the major finding was that the Age × Series interaction was statistically significant, F (4, 216) = 4.79, $p < .01$. Although aged rats ran to the food reward faster than young rats in the initial training series, at the last training series young rats ran to the food reward significantly faster than aged rats.

During test trials, the major findings were for the error scores and concerned the highly significant age main effect, F (1, 52) = 53.94, $p < .01$. Aged rats made significantly fewer errors than did young rats. An age × Series interaction (F (3, 156) = 36.82, $p < .01$) occurred because age differences were greater on the initial series of trials than on the last series of trials, although aged rats made fewer errors than young rats for all four series. During massed practice, young rats had significantly higher error scores the first trial on a day following a series, while the mean error scores of aged rats did not increase for these comparisons, a finding which may explain a significant 3rd order interaction of age, practice condition, series, and trials, F (9, 468) = 4.68, $p < .01$.

The procedure of forced correct responding was found to facilitate learning of a complex problem more for aged than mature-young rats. Aged rats made fewer errors than young rats during maze learning tests after forced correct response trials. During the usual test procedures, without forced correct responses, aged rats make more errors than young rats, and about 50% of the aged rats fail to reach a criterion of learning of one error per trial during long sequences of trials. In contrast, the present research demonstrated fast efficient mastery of the complex problem by aged rats. This finding may be related to earlier research in our laboratory. We had previously found that aged rats were more rigid than young rats during maze learning and that aged rats were more rigid than young rats during maze learning and that aged rats engaged in less exploration behavior than young rats. The use of massed practice coupled with assistance in making correct responses early in learning may significantly increase learning efficiency of aged animals and aged people.

2. Rats were trained on a continuous reward schedule in a 2-bar test box with or without light contingency associated with food reward. During extinction trials with the light contingency presented on continuous reward, the light contingency was shown to facilitate extinction on the basis of response totals (energizing effect) and percentage of responses on the reward bar (directional effect). For these extinction trials, no evidence was obtained for secondary reinforcing properties of the light contingency. However, a second experiment found a secondary reward energizing effect when the light contingency was presented on fixed ratio schedules during extinction trials (see Table 1). These energizing effects also were obtained for rats trained without the light contingency, but to a lesser degree than for rats trained with the light contingency. A moderately durable energizing effect of secondary reward was obtained which was an increasing function of partial periodic secondary reward during extinction trials; i.e., responses were highest for fixed ratio with every tenth response followed by light, next highest with every fifth response followed by light, and last with no light rewards. Presentation of the light on a fixed ratio schedule during extinction trials also resulted in a strong directional effect for all groups regardless of the training condition; i.e., a large proportion of the total responses were on the previously food rewarded bar. The implication of this research are that cues unrelated to primary reward may be utilized to sustain behavioral sequences related to learning or work related behavior. Studies of the relations of primary and secondary reward schedules to learning and retention during long retention intervals are of basic importance in the study of the aging process.

Significance to Bio-Medical Research and the Program of the Institute: Learning and/or memory deficits represent a major problem among the aged human population. Major behavioral techniques to reduce performance deficits obtained for aged, lower animals have been studied in our laboratory. This project may facilitate research with higher organisms such as man by determining the optimal conditions for learning and for retention of learned responses.

Proposed Course of Project: Further studies are in progress to determine the nature of the partial reinforcement effect in relation to (a) time contingent vs. response contingent partial reinforcement, and (b) massed vs. distributed extinction trials. Other studies will examine age differences in operant performance as a function of response effortfulness. Maze studies will concentrate upon the effects of central nervous system stimulants on behavioral rigidity within the maze for old rats. We will also initiate preliminary studies of rote learning and perceptual learning of human subjects.

Honors and awards: None

Publications: Goodrick, C. Maze learning of mature-young and aged rats as a function of distribution of practice. Journal of Experimental Psychology, 1973, 98, 344-349.

Goodrick, C. Primary and secondary motivational properties of a light stimulus. Psychological Reports, In Press.

	TRAINED WITH LIGHT			TRAINED WITHOUGHT LIGHT		
TRIALS	NLC	LC-FR5	LC-FR 10	NLC	LC-FR5	LC-FR 10
1	127	157	168	140	205	178
2	58	80	118	65	87	90
3	20	77	98	26	52	55
4	20	62	108	25	43	45
5	22	64	81	20	40	38
6	17	61	117	17	40	39

TABLE 1 - Mean responses (energizing effect) of rats during extinction trials as a function of training conditions and extinction conditions. (NLC, no light contingency; LC-FR5, light contingency, fixed ratio 5; LC-FR 10, light contingency, fixed ratio 10.)

Serial No. HD-LBS-10
1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Learning and Problem Solving Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 throught June 30, 1974

Project Title: Exercise, general activity level and aging

Previous Serial Number: HD-AG 27

Principal Investigator: Charles L. Goodrick, Ph.D. (30%)

Other Investigator: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	.60
Professional:	.30
Others:	.30

Project Description:

Objectives: The general objectives are: (1) to determine methods for increasing vigorous physical activity of lower animals during late stages in the life span (2) to examine behavioral and longevity differences among animals which differ in physical activity level, and (3) to determine the physiological mechanisms underlying differences in activity.

Methods Employed: Wistar rats or various strains of mice are placed in standard activity wheels and allowed access to free voluntary exercise. Hungry animals also may be rewarded with food for running. Other studies utilize inbred, hybrid, and mutant mice or species which differ in activity level due to different genetic constitutions. (See project HD-LBS 1 Behavioral genetics and aging).

Major Findings:

A. We currently have a major investigation in progress using rats to determine the effects of socialization during wheel exercise upon longevity, body weight, food intake, and metabolic rate. Preliminary data are in the process of analysis and will be reported in detail next year. The major finding, based on preliminary examination of the wheel scores, is a significantly increased level of wheel exercise for both male and female rats later in the life span, when the rats are paired rather than singly tested in the wheels.

One of the important differences between wheel exercise groups and control groups concerns differences in body weight. Male rats paired during exercise had lower body weights than male rats isolated during exercise or control male rats. Female rats (isolated or paired in exercise wheels) had lower body weights than control female rats not allowed wheel exercise. The groups (both male and female) with low body weight had high scores of wheel activity compared with the groups with relatively high body weight. At 145 days of age, randomly selected isolated and paired rats (both male and female) were removed from the exercise wheels and naive rats the same age were placed in the exercise wheels. These groups were compared with respect to body weight gain and wheel exercise over a 40 day interval, and food intake also was measured during days 30-40 following initiation of these procedures.

The food intake of rats, both male and female, which were removed from the wheels was greater than that of controls not previously given access to wheel exercise. Groups most active during the wheel exercise tests (male paired, female isolated, female paired) had higher food intake scores than control groups. These most active groups also had lower body weight gain scores than control groups.

A major finding concerned the body weight gain of the three most active groups placed in wheel exercise tests at maturity when 145 days old. These groups had a mean net reduction of body weight, (although an increment in food intake) compared with control groups. By the end of the 40 day block of wheel exercise tests, the activity level of the three most active groups was similar to that of rats given wheel exercise when 45 days old.

The following conclusions are based on the above data. Moderate voluntary exercise during growth results in lower body weight, despite greater food intake, for both male and female groups, compared with control groups not allowed access to voluntary exercise test units. Animals which initiate voluntary exercise later in development show a striking cessation of body weight gain compared with controls never allowed exercise or controls allowed exercise since early development. Animals which are raised in a social unit participate more in voluntary exercise than do singly raised subjects.

B. Mice are nocturnal animals that are highly active at night. A series of experiments concerned with the effects of age and lighting conditions upon the activity and periodicity of young and aged mice were performed. The mice were either maintained under standard regimens of 12 hours light-12 hours dark (controls) or allowed to produce their own lighting schedules by manipulating lever switches in their cages (experimentals). The results show that experimental aged mice turned their lights on in a darkened environment significantly more than did the young mice. Since other data show that young mice engage in more runwheel activity than do old mice, an inverse relationship was postulated between light preference and runwheel activity. A test of this relationship revealed that when activity wheels were added to the control and experimental environments, most of the wheel activity occurred during time periods in which lights were off regardless of group or age of mice. The runwheel activity patterns of young and old mice in these studies revealed that the periodicities of the young animals were much closer to 24 hours between activity onsets than was the case for the old mice, whose periodicities were somewhat greater than 24 hours.

Significance to Bio-Medical Research and the Program of the Institute: One of the consistent findings of gerontological research is the decline in general activity level of old animals compared with young animals. It is important to determine whether quantity of activity (e.g. wheel activity) and/or quality of activity (e.g. increased exploration behavior or greater response variability) may be increased experimentally for old and senescent animals. It is also important to examine the role of heredity with respect to voluntary exercise throughout the entire lifespan, and the effect of exercise upon behavioral decrements associated with advanced old age. The knowledge and utilization of factors which change base activity levels of aged animals may result in more productive later years for aged humans.

Proposed course of the project: The studies of rat wheel exercise will continue to determine the effects of voluntary exercise upon longevity for paired rats, and to determine the amount of voluntary exercise during advanced old age.

Studies of wheel exercise periodicity of young and aged mice will be continued. Periodicity patterns of mice will be examined throughout old age. Additional studies will determine the level of voluntary activity for young and aged mice which are allowed voluntary control of lighting conditions within the home environment.

Honors and Awards: None

Publications: Goodrick, C. The effects of exercise on longevity and behavior of hybrid mice which differ in coat color. Journal of Gerontology, 1974, 29, 129-133.

Goodrick, C. Exploration behavior of immature albino rats. Developmental Psychology, In Press.

NICHD Annual Report
July 1, 1973 through June 30, 1974
Gerontology Research Center
Laboratory of Cellular and Comparative Physiology

Research activities have continued along the goals set forth a year ago, with particular emphasis on how aging modulates the proliferation and differentiation processes at the cellular and molecular levels. Several model systems are under investigation, ranging from mammals to invertebrates, and the results are discussed beginning with the immune system--the focus of research activities in this laboratory.

Studies on the effects of aging on the immune system are centered about 3 areas: (a) cellular and functional properties of subpopulations of lymphoid cells, (b) effects of aging on the modulation of proliferation and differentiation of immunocompetent cells and (c) characterization of artificially induced and naturally occurring immunodeficiency states. During the past year many exciting findings have been obtained in these areas of research and some of them are described in the above order.

A. Cellular and functional properties of subpopulations of lymphoid cells.

(1) While there is no appreciable shift in the number and proportion of the major cell types, thymus-derived (T) cells, bone marrow-derived (B) cells, and accessory (A) cells, in the spleen cells of mice with age, the proportion of the less dense T-mitogen unresponsive cells is increased at the expense of the denser T-mitogen responsive cells. One possibility is that the less dense cells are being selected with age and are less dense due to a change in the nuclear-cytoplasm ratio.

(2) The DNA synthetic activity of mitogen stimulated spleen T cells declines with age, but not that of mitogen stimulated spleen B cells, suggesting that there may be an unbalanced shift with age in subpopulations of lymphocytes, and as such a possible unbalanced shift in immune functions.

(3) Age-related decline in DNA synthetic activity of T cells is correlated most strikingly with the level of cyclic-GMP. Because changes in the level of cyclic nucleotides can be detected within minutes after mitogen stimulation and long before the onset of DNA synthesis and because there is evidence that changes in the intracellular levels of the cyclic nucleotides initiate and regulate cellular proliferation and differentiation, these results suggest that profound changes may be occurring at the membrane level with age.

(4) Subpopulation of spleen lymphocytes have different sensitivity to X-irradiation, mitomycin treatment and freezing rates. This suggests that selective subpopulations of lymphocytes can be prepared through simple treatment of the cells and that the *in vitro* response to antigen or mitogen can be categorized according to what type of cell is responding on the basis of the sensitivity to these various treatments.

B. Effects of aging on the modulation of proliferation and differentiation of immunocompetent cells.

(1) A humoral nonantigen-specific factor derived from human peripheral lymphocytes is capable of inducing an enhanced antibody response of T cell deficient mouse spleen cells *in vitro*.

(2) Thymus cells are normally unresponsive to bacterial lipopolysaccharide (LPS), a B cell-specific mitogen, but treatment of thymus cells with concanavalin A, a T cell mitogen, can influence LPS-responsive cells to emerge.

(3) Thymic epithelial cells of old mice, in contrast to those of young mice, when implanted into T cell-deficient young mice appear to be incapable of transforming precursor cells into functional T cells. Whether or not this age-related defect is irreversible or reversible remains to be resolved.

(4) Dietary studies show that (a) perinatal protein undernutrition does not affect the thymus as much as other organs, suggesting that mechanisms of regulation of its growth may be different from those of other organs, and (b) protein undernutrition of young adult mice can affect both the rate of cellular differentiation and retention of immunologic memory cells.

C. Characterization of artificially induced and naturally occurring immunodeficiency states.

(1) Studies on allogeneic chimeric spleens show that they lack a subpopulation of T cells, and the deficiency can be replaced by a nonspecific human lymphocyte factor *in vitro*. A "spin off" of this series of studies is the demonstration that a laminar flow tent is as effective as a germ-free room in the rearing of immunosuppressed and immunodeficient animals (e.g., very old animals) at a cost and effort considerably less than it would take to maintain them in a germ-free room.

(2) In autoimmune-prone NZB mice, the cell-mediated immune activity is decreased in all old males, but that of old females was highly variable.

(3) Morphological, clinical and immunological analyses of the kidney lesion of Wistar rats revealed that (a) its onset occurs as early as 3 months of age, (b) most of the old rats manifest the lesion (male, 100%; female, 80%) and (c) about 90% of the males and 50% of the females die of the lesion. These results show that these rats are not suitable animal models to study aging of certain physiological functions.

Studies on the role of maternal age on the frequency of chromosomal aneuploid progenies have been initiated recently with the objective of developing the mouse as an animal model. Encouraging preliminary results have been obtained; e.g., significant numbers of chromosomal aneuploid progenies were observed of retired breeders. In addition, a significant increase in preimplantation death of embryos was noted in the autoimmune-prone NZB strain of mouse. Based on these results we are cautiously optimistic that many complex problems on "maternal age effects" in the human can be approached systematically in the mouse.

Research on the mechanisms of in vitro cellular aging analyzes what cytokinetic and biochemical events become limiting as different types of human cells age in vitro. Three exciting findings have been made. (1) Aneuploid cell cultures derived from trisomic-21 patients have a decreased rate of cell replication in comparison to normal diploid cells at the same early passage. This may be responsible for their short "life span." (2) The high RNA content of late passaged WI-38 cell cultures appear to be reflective of accumulation of RNA by the nondividing subpopulation of cells and not to altered RNA synthetic pathways, contrary to the expectation of many. (3) A striking difference in various cytokinetic indices exists between lung and skin fibroblasts obtained from the same embryo. The lung fibroblasts were similar to the early passaged Hayflick WI-38 (lung) cells, whereas those from the skin resembled late passaged WI-38 cells. This discovery emphasizes the danger in equating lung and skin fibroblasts, as done commonly by many, and that caution should be taken in the use of certain in vitro population aging parameters as reflective of functional aging at the individual cellular level.

Other studies revealed the following. (1) Massive synthesis of poly(A), which is presumed to have a regulatory role in message metabolism, occurs only during the initial inception of embryogenesis; postfertilization polyadenylation is not altered by inhibitors of DNA-directed RNA synthesis, unlike adult somatic cells; and latent genetic codes are localized in the subribosomal fractions of the ovum, and fertilization rapidly transposes the adenylated genetic messages into the ribosomal fraction. (2) Steroid binding sites in the cytosol appear to decrease with age in various tissues and organs. (3) Rodents can tolerate a diet with a relatively low protein content once they reach adulthood. (4) Age-related changes associated with lysosomal degradation can be detected in papillary myocardial cells.

The Laboratory of Cellular and Comparative Physiology has completed its second year under a new set of goals directed toward an understanding of the effects of aging on regulation of proliferation and differentiation. Associated with the changes in the laboratory goals, there has been a change in research approach to understand biologic mechanisms at the expense of biologic phenomena. This has necessitated a change initiated with the beginning of FY 1974 in the organizational structure of the Laboratory in an attempt to create a scientific atmosphere conducive for productive interaction among the investigators. In view of these changes we are now looking forward to the coming years with greater enthusiasm and optimism.

Serial No.: HD-CCP-1
1. Gerontology Research Center
2. Laboratory of Cellular and
   Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Changes in Cells and Extracellular Matrices of the Crayfish Gastrolith Disc during Growth, Development and Aging

Previous Serial Number: Same

Principal Investigator: Dorothy F. Travis

Other Investigators: None

Cooperating Units: None

Project Description: This project has been discontinued.

Honors and Awards: Dr. Travis serves as an Advisory Editor to the journal, Calcified Tissue Research.

Publications: None

Serial No.: HD-CCP-2
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Dietary Proteins and Longevity

Previous Serial Number: Same

Principal Investigator: C. H. Barrows, Jr.

Other Investigators: None

Cooperating Units: None

Project Description: This project has been discontinued.

Publications: None

Serial No.: HD-CCP-8
1. Gerontology Research Center
2. Laboratory of Cellular and
Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Age-Related Changes in Mammalian Myocardial Cells

Previous Serial Number: Same

Principal Investigator: Dorothy F. Travis

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.0
Professional:	1.0
Others:	0.0

Project Description:

Objectives: The broad objectives of this program have been directed at understanding basic structural and functional principles underlying the activities of myocardial cells during developmental sequences and aging process. Encompassed in these considerations of highly differentiated post-mitotic cells, studies have been carried out on both left ventricular mural and papillary myocardial cells.

Methods Employed: Ultrastructural and electron cytochemical, both modified and non-modified procedures, have been used in these studies of 6, 12, and 24 month old male rats.

Major Findings:
A. Age related changes in the rat left ventricular papillary myocardial cells.

1. Changes in mechanisms of lysosomal degradation. Results of investigations of the left ventricular papillary myocardial cells of the rat, dealing only with changes in regions of the muscles with relatively normal tissue architecture, have shown that pronounced age-related changes occur. Qualitative, as well as quantitative changes, were evident in papillary myocardial cells which contrasted with less marked changes reported from this laboratory in cells of the left ventricular free wall. Three previously unreported qualitative age-related changes occurred: a) Nuclear changes were exemplified by marked lobulations and an increase in euchromatin with a concomitant

reduction in heterochromatin, modifications related to increased protein synthesis. b) Mitochondrial changes in the nuclear pole zone were exemplified by swelling and clarification of their matrices, which resemble those resulting from hypoxia. c) Changes in mechanisms of autophagic cytomembrane segregation. Although the usual 1 or 2 ER-derived membrane pairs were commonly involved in segregation of mitochondria in both young and old cells, a conspicuous variation of this occurred only in old cells, where ER-derived multilamellar membranous sheets segregated mitochondria. Deteriorating mitochondria, while still surrounded by seemingly redundant membranes whose cisternae show reactivity for acid hydrolases, suggest that hydrolytic enzymes may be delivered by this direct route to the interior of the vacuoles in old cells. Qualitative changes were also evident in lipid droplets which showed moderate to marked increases in electron density, reflecting a decrease in preparative solubility and suggesting an apparent change in the nature of the lipid with age. Quantitative age-related differences in the cells that were commonly observed included: lipid droplets segregated by ER-derived membranes, rarely evident in young cells; what was interpreted as an increased production of two types of primary lysosomes; marked increases in secondary lysosomes containing mitochondria and lipid droplets in various stages of degradation; and increases in the size and number of residual bodies. Although the excretion of solid wastes does not cease completely in senescent papillary myocardial cells, neither the rate of degradation nor excretion of residue could be proved to account for the observed increases in lysosomes and residual bodies. That qualitative differences in cellular degradative phenomena with age might be responsible is suggested from changes in mechanisms of autophagic segregation of mitochondria by seemingly redundant membranes in the old cell. This manuscript is prepared for review and will be submitted to the Journal of Ultrastructure Research for publication with the manuscript now in preparation, relating to degenerative changes in papillary myocardial cells.

2. Degenerative changes. The results from this study have indicated that further marked qualitative and quantitative age-related changes occur in old cells of the left ventricular papillary myocardium which lead to cell death and consequently to extensive regions of the muscle in which necrosis and tissue fibrosis are pronounced. The further qualitative changes were evident in the nucleus, endoplasmic reticulum, mitochondria, the T-system, intercalated discs, contractile elements, sarcoplasmic matrix, and sarcolemma. The most marked quantitative changes were an increased production of primary lysosomes but a reduction in both secondary lysosomes and residual bodies, a decrease in lipid droplets, a loss of myocardial cells and thus contractile elements, an increase in phagocytic cells, fibroblasts, and deposited collagen. These changes in old papillary myocardial cells from regions of the papillary muscles of grossly abnormal tissue architecture contrasted markedly from those regions showing relatively normal tissue architecture, but in which the old 24 month cells were clearly altered but more subtly when compared to the 6 and 12 month cells. This manuscript is being prepared and will be submitted to the Journal of Ultrastructure Research.

Proposed Course: This project will be terminated.

Honors and Awards: None

Publications: Topping, T.M. and Travis, D.F.: An electron cytochemical study of mechanisms of lysosomal activity in the rat left ventricular mural myocardium. . J. Ultrastruc. Res. 46: 1-22, 1974.

Serial No.: HD-CCP-11
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Ultrastructural Studies of the Regenerating Sea Urchin Spine During Growth and Developmental Sequences

Previous Serial Number: Same

Principal Investigators: Barry M. Heatfield
Dorothy F. Travis

Other Investigators: None

Cooperating Units: None

Project Description: This project has been discontinued.

Serial No.: HD-CCP-14
1. Gerontology Research Center
2. Laboratory of Cellular and
   Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Cellular Basis of Regulation of the Humoral Immune Response

Previous Serial Number: Same

Principal Investigator: Albert A. Nordin

Other Investigator: Barbara E. Loughman

Cooperating Units: None

Man Years:

Total:	1.9
Professional:	1.0
Others:	0.9

Project Description:

Objectives: The goal of this project is to characterize the regulation of the immune response by cellular elements. Efforts to determine the origin and mechanism of action of these cells are of prime interest.

Methods Employed:

1) B-cell source-Young C57Bl/6 mice (6 weeks of age) are thymectomized, irradiated 2 weeks later and grafted with 10 x 10^6 syngeneic bone marrow cells 24 hours after irradiation. Four to eight weeks later the spleens of these mice serve as a source of bone marrow derived lymphocytes (B-cells).

2) T-cell source-six to eight week of C57Bl/6 or $B_6D_2F_1$ mice are used as donors for thymus glands. X-irradiated (850r) syngeneic mice are injected with 50-70 x 10^6 thymus cells with or without a simultaneous injection of antigen. Seven or eight days later the spleens of these mice are used as a source of thymus derived lymphocytes (T-cells). Those cells from mice injected with thymocytes and antigen are referrec to as activated T-cells and those from mice receiving only thymocytes are referred to as normal T-cells.

3) Adherent cells from carbonyl iron treated spleen-After treating spleen cells with carbonyl iron, the remaining population is plated in plastic dishes and incubated for one hour. Non adherent cells are

removed and the adherent layer washed exhaustively. The adherent cells are removed by trypsin in a viable state.

4) The *in vitro* culture technique and the assay for plaque-forming cells are routine methods.

Major Findings:

1) A single preparation of activated T-cells (ATC_{SRC}) can either suppress or enhance the *in vitro* immune response. Enhancement or partial reconstitution of T depleted cell populations is observed with low (0.5 x 10^6) numbers of ATC_{SRC} whereas high (2 x 10^6) numbers of the same ATC_{SRC} preparation significantly suppress the immune response.

2) The suppressive effects of ATC_{SRC} is not antigen specific in that the immune response to burro red cells is effectively suppressed by the addition of ATC_{SRC}. Furthermore unactivated T-cells suppress the *in vitro* immune response as efficiently as ATC_{SRC}.

3) The regulatory activities of ATC_{SRC} reside in the population of cells which is non-adherent and sensitive to anti θ.

4) The following cell preparations were assayed as sources of suppressive and/or enhancing cell populations.
   a) Cortisone resistant thymocytes were used in place of thymocytes for preparing ATC_{SRC}.
   b) Spleen cells from lethally irradiated mice that were either injected with only SRC or left uninjected and cells collected 8 days post-irradiation.
   c) Thymocytes with or without SRC were placed in cell-impermeable chambers and put into the peritoneal cavity of lethally irradiated mice for 8 days.
   d) Spleen from the recipient mice in (c) were harvested at the same time the chambers were removed.

   All preparations, except one, showed suppressive activity when cocultivated with normal spleen cells. The exception concerns the thymocytes which were incubated in chambers for 8 days without SRC. These cells markedly enhanced the response of normal spleen cells when 0.25 x 10^6 were added but markedly suppressed when 1.0 x 10^6 were added. The enhancing properties of these cell preparations were assayed with either spleen cells from thymectomized-irradiated-bone marrow reconstituted mice or with normal adherent cells as sources of B cells. Not all cell preparations were assayed for enhancement but in general only those cell preparations which were prepared in the presence of SRC were active. The one exception was again the thymocytes in the peritoneal chamber without SRC. These cells were able to enhance normal adherent cells.

5) Treatment of normal spleen cells with carbonyl iron to remove phagocytic cells results in a preparation which is inactive in the

in-vitro primary immune response. However, cocultivation of such preparations with irradiated adherent cells to supply the third cell type does not completely restore the full capacity to respond in vitro.

6) Adherent cells prepared from carbonyl iron treated spleen cells are for the vast majority lymphocytes. These adherent lymphocytes respond markedly well to LPS but do not respond to PHA.

7) Removal of adherent cells from carbonyl iron treated spleen cells results in a preparation of cells which consistently respond better than carbonyl iron treated spleen cells.

Significance to Biomedical Research and the Program of the Institute:
The goal of this research program is to examine the cellular populations that are regulating the humoral immune response. The mechanisms by which the regulation takesplace would be of significance not only to the field of immunology but would have relevance to cell biology. It is also significant to the area of immunosenescence. The decline in immunological responsiveness with age is well established but the reasons are not at all understood. The role of regulatory mechanisms in explaining the phenomena of immunosenescence may be of considerable significance.

Proposed Course:

1) Investigate the occurrence and distribution of naturally occurring suppressor cells.

2) Continue the studies on the attempts to separate, identify and characterize cells carrying out regulatory events.

3) To establish if suppressive effects are mediated by soluble factors.

4) Initiate investigations on the occurrence and importance of suppressor cells when old animals are available.

Honors and Awards: Dr. Albert A. Nordin is presently a member of the Council of the Regulatory Biology Section of the National Science Foundation.

Publications: Loughman, B.E., Farrar, J.J., and Nordin, A.A.: Presence of antibody-forming cell precursors in the mouse adherent spleen cell population. J. Immunol. 112: 430-432, 1974.

Serial No.: HD-CCP-15
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Cellular Aspects of the Immune Response of Long-term Radiation Induced Allogeneic Chimeras

Previous Serial Number: Same

Principal Investigator: Barbara E. Loughman

Other Investigator: Albert A. Nordin

Cooperating Units:

Man Years:

Total:	1.2
Professional:	0.7
Other:	0.5

Project Description:

Objectives: The major objective of the proposed research is to examine the immune system of x-irradiated allogeneic bone marrow grafted mice. These allogeneic radiation chimeras serve as a model for investigating the basic cellular aspects of immune mechanisms with particular emphasis on regulatory mechanisms involved in immunologic responsiveness. Another objective is to use these allogeneic radiation chimeras as well as aggregation chimeras (tetraparental-mice) to determine the effect of somatic tissue-immature lymphoid cell interactions on the lymphopoietic differentiation process.

Methods Employed: Previous work has established the fact that allogeneic bone marrow chimeric mice can only survive the secondary disease effects of a graft-versus-host (GVH) reaction in a controlled germfree environment. However, there is reasonable evidence to suggest that the clean environment provided by a laminar flow animal facility will also promote long-term survival. Animals quartered in a laminar flow room are given a low lethal dose of x-irradiation followed by a bone marrow graft from either a syngeneic or allogeneic donor. The survival of these animals will be followed. Once it has been established that the clean environment will promote long-term survival the animals will be examined by histological and immunological techniques to determine whether the immunological reconstitutive process is the same as that seen in germfree chimeras. Any subsequently observed immunologic deficiency will be examined to determine the cellular aspects of the deficiency. Experimental attempts to compensate for or eliminate the deficiency will be carried out.

Major Findings:

1. Survivorship curves of both syngeneic mouse radiation chimeras (C3H/He → C3H/He, DBA/2 → DBA/2, C57Bl/6 → C57Bl/6) and allogeneic mouse radiation chimeras (DBA/2 → C3H/He, DBA/2 → C57Bl/6, C3H/He → DBA/2) indicate that animals which have been in the controlled environment for 2 weeks prior to treatment have a 3.5 month post transplant (p.t.) survival of greater than 80%. These animals are hematopoietically reconstituted (few deaths 7-14 days p.t.) with few dying from acute infection of pre-treatment etiology immediately after treatment (0-8 days p.t.). If the animals are quarantined for at least two weeks in the laminar flow tent prior to treatment, remarkably few animals succumb to the secondary disease of a GVH reaction. This is confirmed by the fact that greater than 60% of the animals treated with x-irradiation and a bone marrow graft prior to laminar flow tent stabilization succumb 14 to 34 days p.t.

2. Allogeneic chimeras (3-9 months p.t.) have been checked for lymphoid cell H-2 type with the use of isoantisera. These studies have confirmed the fact that as with the germfree chimera these animals have lymphoid elements which are of donor origin.

3. Flourescent antibody studies have shown that these animals do have theta antigen bearing cells in their spleen. The syngeneic and allogeneic chimeras have comparable numbers of theta positive cells although the number of these cells found 7-9 months p.t. is only about one-third the number found in normal spleen cells.

4. Humoral immune responsiveness of these allogeneic radiation chimeras is depressed to the same extent as was seen with germfree allogeneic chimeras. The depression is seen after either in vivo or in vitro stimulation with sheep erythrocytes (SRC).

5. Two types of experimental approaches to in vitro reconstitution of this depressed humoral response have been done. Activated thymus-derived lymphocytes (ATC_{SRC}) from either a syngeneic or allogeneic source have been used. To date the use of syngeneic ATC has not been successful although the use of allogeneic ATC_{SRC} was. Human peripheral blood lymphocytes (HPL) were used as a source of xenogeneic thymus-derived cells. The use of low numbers of HPL fully restored the responsiveness of allogeneic chimera spleen cells to SRC.

6. Attempts to reconstitute the in vivo responsiveness to SRC with the use of HPL were not successful.

7. Allogeneic chimera spleen cells have been checked for their differential reactivity to mitogens in an attempt to determine the differentiation state of the spleen lymphoid elements. Normal DBA/2 spleen cells are reactive to phytohemagglutinins (PHA-mature T-cell mitogen), bacterial lipopolysaccharide (LPS-B-cell mitogen) and staphyloccocus enterotoxin B (SEB-T-cell mitogen which may stimulate both mature and immature T-cells). Spleen cells from DBA→ C3H/He allogeneic chimeras are responsive to LPS and SEB but are refractory to PHA.

8. The *in vitro* cell-mediated immune response to third party of the allogeneic radiation chimera spleen cells is not comparable to that seen with normal spleen cells.

Significance to Biomedical Research and the Program of the Institute:

The results of these investigations will be significant in a practical sense. Clinical bone marrow grafting within a heterogeneous population has necessarily been restricted mainly as a result of immunologic damage to the host. For obvious reasons, the parameters involved in these reactions are not as easily approached in humans as they are in a representative animal model. Techniques are available to experimentally determine not only the favorable conditions, which permit the establishing of allogeneic bone marrow chimeric mice, but also to define the events resulting in immunoregulation observed in such animals. Hopefully, these results will bear a resemblance to those encountered in human bone marrow grafting. Information may then be available which will reduce the risks in bone marrow grafting and result in the reconstitution of an intact immune mechanism.

Basic information pertinent to the immune response is equally significant. Cellular immunology, during recent years, revolved around cell cooperation as a basic requisite for the expression of antibody to many antigens. Although the majority of such studies has considered the interactions between thymus dependent and bone marrow dependent cells, it is becoming increasingly obvious that other cell type cooperative efforts are involved in the complex immune systems of mammals. The deficiency of any one cell type most likely results in regulating the expression of immunity involving several lymphoid elements. The information which can be supplied in attempts to re-establish immunological competence to the allogeneic chimeric mice is expected to demonstrate and define the importance of cellular interactions in both the humoral and cell-mediated forms of the immune response.

Studies concerned with immunodeficiency diseases will be of significance since many naturally occurring examples of such diseases have traced a causative factor(s) to the thymus gland. This model system with a well defined thymic defect should provide meaningful data relevant to the immunodeficiency disease state.

Proposed Course: The proposed course is to continue with plans contingent on the ability of the laminar flow environment to prolong the life of allogeneic radiation chimeras with particular emphasis on the immunologic function of the splenic and thymic cellular components of the chimera. Of most immediate interest is to follow up on the observation that the theta-positive spleen cells in the allogeneic radiation chimera seem to be in a different state of differentiation than those seen in normal spleen cell populations (mitogen experiments). Also experiments will be initiated to examine the role of thymic epithelium on the differentiation of thymus-cell precursors into functional thymus-derived lymphocytes. These experiments will be carried out using neonatally, thymectomized, tetraparental mice which have been given a thymic graft of parental origin.

Publications: None

Serial No.: HD-CCP-16
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: *In Vitro* Studies on the Capacity of Aged Mice to Develop Immunological Memory

Previous Serial Number: Same

Principal Investigator: John J. Farrar

Other Investigators: T. Makinodan
B. F. Loughman
A. A. Nordin

Cooperating Units: None

Man Years:

Total:	1.7
Professional:	1.3
Other:	0.4

Project Description:

Objectives: The objective of this research is to define the specific memory cell deficiencies in the aged mouse. A variety of reports have shown that T-lymphocytes from primed animals when challenged with the same antigen release a soluble factor(s) which enhances antibody responses to unrelated antigens. Exaggerated primary antibody responses may be induced by these active factors. The factors therefore may be T-cell-derived mediators of the enhanced antibody production of the secondary or memory response. Although these factors have not been detected following primary immunization of T-cells with routine antigens, they may be "artificially" induced in mixed lymphocyte cultures (MLC) and with mitogen stimulation. A study of MLC and mitogen induced stimulation of T-lymphocytes therefore provides an experimental model for studying the active factors derived from antigen-primed T-cells.

Methods Employed: Modification of the *in vitro* immunization procedure described by Mishell and Dutton and the method of Marbrooke were used throughout the work. As employed, the systems were used to culture and immunize spleen cells from thymectomized, irradiated, and bone marrow reconstituted mice. Some of the cultures were supplemented with xenogeneic (human) T-lymphocytes. The generation of antibody-forming cells (AFC) was assessed by the Jerne and Nordin hemolytic plaque forming cell assay.

Major Findings: The results of the experiments conducted during the past year have suggested that a non-antigen-specific mediator derived from human peripheral lymphocytes (HPL) is capable of inducing enhanced AFC responses from T-cell deficient mouse spleen cells. The magnitude of the responses are comparable to the responses obtained from specific antigen-primed normal mouse spleen cells.

Regression analysis of HPL dose response experiments in the xenogeneic system suggested that the HPL were contributing a single reconstituting cellular function to the T-cell deficient mouse spleen cell cultures. The reconstituting cells in the HPL population were shown to be resistant to doses of x-irradiation up to 800R and not removed by treatment with carbonyl-iron and a magnet which is known to remove phagocytic cells. Marbrooke culture vessels with two chambers separated by a 0.2μ porosity membrane were utilized to show: (1) that the HPL mediated the "xenogeneic helper" function via a diffusible factor, and (2) that the AFC response of the HPL is negligible. It was further shown that the HPL "helper" factor was not antigen-specific. Stimulation of the human lymphocytes with allogeneic lymphocytes or activation with various T-cell mitogens (PHA, Con A, or SEB) induced significant amounts of the "helper" factor.

Significance to Biomedical Research and the Program of the Institute: The results of the experiments are significant to the field of biomedical research in that they show that at least one of the non-antigen-specific T-cell derived enhancing factors of the immune response is capable of crossing the species barrier. The importance to the field of gerontological research stems from the fact that the early experiments showed that the secondary antibody (IgM) responses of spleen cells from aged mice were severely depressed in magnitude. The results from the experiments using the xenogeneic system raise the possibility that a contributory defect in the secondary response of aged mice is a failure to develop comparably active T-lymphocytes during primary immunization.

Proposed Course: The proposed course of future experiments will be to determine the molecular nature of the human lymphocyte-derived mediator and to determine its mechanism of action.

Honors and Awards: None

Publications:

Farrar, J.J., Loughman, B.E., and Nordin, A.A.: Lymphopoietic potential of bone marrow cells from aged mice: comparison of the cellular constituents of bone marrow from young and aged mice. J. Immunol. 112: 1244-1249, 1974.

Farrar, J.J.: In vitro reconstitution of T-cell deficient mouse spleen cells by co-cultivation with human lymphocytes. J. Immunol. 112: 1613-1616, 1974.

Serial No.: HD-CCP-17
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Pathology of Age-Associated Kidney Disease of Wistar Rats Reared at GRC

Previous Serial Number: Same

Principal Investigator: Katsuiku Hirokawa

Other Investigator: None

Cooperating Units: None

Man Years:

Total:	0.3
Professional:	0.3
Others:	0.0

Project Description:

Objectives: The objective of this study is to establish the nature of age-related kidney disease of Wistar rats reared at GRC. Attempts were made to determine if the kidney disease is related with other age-associated phenomena such as alterations in the immune activity or hormonal environment.

Methods Employed:
(1) Standard light, electron and immunofluorescent microscopic techniques were used.
(2) Permeability of glomerular basement membrane was examined by immuno-enzyme-histochemical method using peroxidase, beef catalase and ferritin.
(3) Kidney-fixed immunoglobulin was eluted from young and old rats and analyzed by standard immunological methods.

Major Findings:
(1) Kidney disease is one of the major causes of death of Wistar rats reared at the Gerontology Research Center. Postmortem necropsy showed approximately 90% of male and 50% of female rats died of or with kidney disease. In randomly sacrificed rats, kidney disease was found in 100% of the male rats after 13 months and 80% of the female rats after 24 months of age. Evidence of kidney disease was histologically recognized in rats as early as 6 months of age. These early lesions were manifested by a slight increase of cellularity and mesangial matrix in the glomeruli.

With age, the glomerular changes became more pronounced and showed crescent formation, thickening of the basement membrane and hyalinization and marked tubular degeneration.

(2) By fluorescent antibody technique, a slight amount of deposition of IgG was found in glomeruli of 3-6-month-old rats. The IgG deposition increased thereafter with age, showing an almost typical pattern of immune complex glomerulonephritis.

(3) Electron microscopically, fusion of podocytes in glomeruli was first evidenced in 3-month-old rats and increased thereafter. The glomerular basement membrane was thickened evenly along capillary tufts, which is quite different from the lumpy lesions seen commonly in immune complex glomerulonephritis. Abrasion of glomerular epithelial cells was a common finding in old rats.

(4) Permeability of glomerular basement membrane was examined by using different size molecules (horseradish peroxidase, beef catalase and ferritin) and a marked increase in permeability was evidenced in old rats.

(5) Clinically, proteinuria appeared at 3 months of age; hypercholestemia was apparent after one year and hypoalbuminemia was also observed in some rats after 18 months of age. BUN and creatinine rose after 18 months of age. Since these clinical findings are similar to the nephrotic syndrome in humans, the kidney disease of the rats could be classified as chronic immune complex glomerulonephritis with nephrotic syndrome.

(6) Elution of kidney-fixed immunoglobulin revealed a relatively large amount of IgG in the kidney of 24-month-old rats, but no specific antibody was serologically detectable. By fluorescent antibody method using anti-Moloney virus antibody, viral antigen was detected frequently but not always in a glomeruli and along the degenerated tubules of old rats. Eluted kidney-fixed immunoglobulin and sera from bilaterally nephrectomized old rats did not induce or accelerate disease when injected intravenously into 2-month-old rats.

(7) There was no evidence of autoantibody as judged by direct Coombs test, presence of autoantibody against kidney, lung, liver and salivary gland tissue, presence of antinuclear antibody and presence of antibody against saline extract of diet pellet.

Its onset occurs early in adulthood, but its severity and frequency increase with advancing age. The source and type(s) of antigen forming the immune complex remain unknown. It is likely that, rather than one specific antigen, a variety of antigens are involved, including viral antigens through infection. The increase in deposition of immune complex in glomeruli with age may be closely related with the decline in the activity of mesangial cells which are involved in the elimination of immune complexes.

Significance to Biomedical Rese rch and the Program of the Institute: The kidney disease of Wistar rats reared at GRC increases with age in its severity and frequency, and appears to have a close relationship with age-associated decline of immune activity. An understanding of relationship between the decline of immune activity and increased occurrence of the disease in aged animals may help to prevent or ameliorate the disease.

Proposed Course: The activity of glomerular mesangial and epithelial cells to eliminate or degrade macromolecular immune complex in young and old rats is proposed.

Honors and Awards: None

Publications: None

Serial No.: HD-CCP-18
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Effects of Age on Steroid Hormone Binding and Responsiveness in Target Cells and Tissues

Previous Serial Number: Same

Principal Investigator: George S. Roth

Other Investigator: None

Cooperating Units: None

Man Years:

Total: 1.0
Professional: 1.0
Others: 0.0

Project Description:

Objectives: A general characteristic of aging organisms seems to be an altered ability to respond to hormonal stimuli. Evidence from this and other laboratories suggests then a long overlooked possible explanation may lie in age-related changes in hormone uptake and/or binding by target cells and tissues. Efforts are currently underway in an attempt to determine (1) whether specific binding of steroid hormones by target cells and tissues is altered as a function of post-developmental age, and (2) whether such possible alterations are responsible for age associated changes in biochemical responsiveness to these hormones.

Methods Employed: The following systems were investigated; primary cell culture, separation, and fractionation, *in vivo* and *in vitro* assays for steroid hormone binding, enzyme inducibility, cellular viability, and nutrient transport.

Major Findings: Decreases in steroid hormone binding by cytosols of rat skeletal muscle, adipose tissue, prostate gland and brain cerebral hemispheres between adulthood (12-13 mo.) and senescense (24-25 mo.) are due to decreases in the number of specific glucocorticoid binding sites. In an effort to correlate age related changes in hormonal responsiveness with changes in hormone binding in a single system, splenic leukocytes were examined. Splenic leukocytes of senescent rats (24-25 mo.) exhibit a 60% reduction in cortisol induced inhibition of ^{3}H-uridine uptake when compared to mature adult animals (12-14 mo.). The degree of inhibition is directly proportional to cortisol dosage up to 2 x 10^{-6} M. Other laboratories have elucidated the mechanism of this response, involving *de novo* synthesis of a transport inhibitory protein. Specific binding of physiological (nanomolar) concentrations of ^{3}H-cortisol by leukocytic cytosol macromolecules is reduced over 40% in the older animals. Moreover, Scatchard analyses reveal 60% fewer specific glucocorticoid binding sites in the cytosols of cells from the senescent rats. Such analyses were performed *in vitro* at 0° C to eliminate metabolism of steroids. In addition, ^{3}H-dexamethasone was used instead of ^{3}H-cortisol to eliminate binding to plasma (or serum) transcortin. The degree of uridine uptake inhibition at varying glucocorticoid dosages is proportional to the amount of specific glucocorticoid binding to cytoplasmic macromolecules. Furthermore, both cortisol induced uridine uptake inhibition and specific cortisol binding are selectively blocked by progesterone administration. These findings suggest that cortisol induced uridine uptake inhibition requires the specific binding of cortisol to leukocytic cytosol macromolecules. Thus, age-related decrease in specific glucocorticoid binding may be at least partially responsible for decreased responsiveness of senescent rat splenic leukocytes to these hormones.

Significance to Biomedical Research and the Program of the Institute: Impaired ability to respond to environmental change is a much stronger index to physiological senescence than chronological age alone. It is thus important to understand the mechanisms by which age changes in adaptive responsiveness occur. Since many response functions are mediated by hormones, gerontologists can derive great benefit from recent progress of endocrinologists in elucidating the molecular mechanisms of hormone action. The present study proposes to apply such endocrinological knowledge to the gerontological problem of impaired adaptive responsiveness. Investigation of hormone action at the molecular level in target cells and tissues from animals of different ages would seem to be a logical first step to approaching this problem. An understanding of age changes in the biochemical mechanisms of hormone action is essential if there is to be hope of halting, preventing or reversing age related lesions in physiological vitality.

Proposed Course: Studies will attempt to correlate age-related changes in hormonal responsiveness with alterations in hormone binding in various target cell systems. Cell separation by density gradients, columns, etc., as well as use of antisera to selected cell populations, will be employed. Such methods should determine whether age changes in hormone binding and responsiveness are intrinsic to given cell types, or due to changes in the proportions of various cell populations within target tissues. The ultimate goal of this investigation will be the determination of the cellular and biochemical mechanisms responsible for age related changes in responsiveness to hormones.

Honors and Awards:

Dr. Roth attended the Third Annual NICHD Cell Culture Workshop held in Los Alamos, New Mexico, July 22-August 3, 1973.

Dr. Roth was an invited lecturer at the Third FASEB Conference "Biology of Development and Aging" held in Atlantic City, New Jersey, April 8-12, 1974.

Publications:

Roth, G.S. and Adelman, R.C.: Possible changes in tissue sensitivity in the age-dependent stimulation of DNA synthesis *in vivo*. *J. Gerontol.* 28: 298-301, 1973.

Roth, G.S.: Age related changes in specific glucocorticoid binding by steroid responsive tissues of rats. *Endocrinology* 94: 82-90, 1974.

Roth, G.S. and Adelman, R.C.: Age related changes in hormone binding by target cells and tissues; possible role in altered adaptive responsiveness. *Exp. Gerontol.* In press.

Roth, G.S.: Age-related changes in glucocorticoid binding by rat spleen leukocytes: Possible cause of altered adaptive responsiveness. *Fed. Proc.* In press.

Serial No.: HD-CCP-19
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mechanisms of In Vitro Cellular Ageing (formerly entitled Effect of Chromosomal Aneusomy on Cellular Growth, Metabolism and Aging)

Previous Serial Number: Same

Principal Investigator: Edward L. Schneider

Other Investigators: Stuart Shorr
Youji Mitsui (10/1/73 EOD)
Jochen Heine (9/16/73 EOD)

Cooperating Units: Dr. R. Cutler
Department of Molecular Biology
University of Texas-Dallas
Dallas, Texas

Man Years:

Total:	3.8
Professional:	2.5
Others:	1.3

Project Description:

Objectives: Currently the most widely accepted in vitro model for studying cellular ageing compares early and late passage human fetal lung fibroblasts (WI-38). It is vital to determine if the cellular alterations observed in late passage or "senescent" fetal lung fibroblasts accurately reflect in vivo human cellular ageing. Therefore we have initiated studies of alternate models of in vitro human cellular ageing: skin fibroblast cultures at early in vitro passage derived from young and old normal human donors, skin fibroblast cultures derived from patients with chromosomal aneuploid conditions, and skin fibroblast cultures derived from patients with other human genetic disorders which feature accelerated in vivo or in vitro ageing. Initial studies were focused on cell replication and nucleic acid metabolism, important cellular parameters which are impaired in in vivo cellular ageing.

Methods Employed: 1) Fibroblast and lymphocyte cultures are obtained from young and old human donors, patients with human chromosomal aneuploid disorders (Down's syndrome), patients with progeria (Hutchison-Guilford syndrome), Werner's syndrome and other disorders which feature accelerated ageing, and from age-matched control human donors. 2) Cell population replication is being measured by the method of DNA dilution and the percent of replicating cells in the cell population is being determined by autoradiography. From these two indices the rate of replication of the replicating cells can be determined. 3) RNA metabolism was analyzed by measuring cellular RNA content, determining the rate of incorporation of exogenous radioactive uridine into cellular RNA, examining the distribution of RNA species by polyacrylamide gel electrophoresis (PAGE), and studying the processing of RNA species by pulse-chase isotope labeling of cell cultures followed by PAGE of their extracted cellular RNA.

Major Findings: Before studies are initiated on skin fibroblasts from young and old donors, it was necessary to compare the properties of these skin fibroblasts to the lung fibroblasts (WI-38) that are commonly employed in *in vitro* ageing. Our preliminary results on fetal lung and skin fibroblast cultures derived from the same fetus and examined at the same early *in vitro* passage reveal a decreased rate of cell population replication, increased cell size, increased RNA content, and decreased tritiated-thymidine incorporation into DNA in the fetal skin cultures. Therefore these skin fibroblast cultures, even at early *in vitro* passage have many of the characteristics of late passage or "senescent" fetal lung fibroblasts (WI-38). These results not only question what are the appropriate parameters for measuring *in vitro* cellular ageing but also indicate the difficulty in equating results derived from human adult skin fibroblasts with those obtained from WI-38 cells.

Spontaneously aneuploid (trisomic-21) skin fibroblast cultures derived from patients with Down's syndrome were demonstrated to have a decreased rate of cell population replication when compared to cell cultures obtained from age-matched controls. Previous studies have demonstrated that these trisomic-21 cell cultures have a decreased *in vitro* lifespan. Utilizing colchicine treatment, aneuploid cells have been created from diploid cells. These induced aneuploid cells are being cloned and the resultant cultures will be used for parallel studies with spontaneous aneuploid cultures.

Cellular protein and RNA, measured per cell number or cellular DNA, increase as cultured human diploid cells (WI-38) enter late *in vitro* passage. This increase is not gradual but appears to occur abruptly after 40 *in vitro* passages. The protein to RNA ratio remains relatively constant reflecting the concomitant nature of the increase in cellular protein and RNA. PAGE analysis of cellular RNA from late passage or "senescent" cells indicates a non-specific increase in ribosomal and transfer RNA in these cells. Pulse-chase experiments revealed that the processing of ribosomal RNA is unaltered in these late passage cells. These results suggest that many macromolecular (RNA) pathways are unimpaired in human diploid cells as they reach the end of their *in vitro* lifespan. The accumulation of macromolecules (RNA and protein) may thus be secondary to their accumulation in non-replicating cells.

Significance to Biomedical Research and the Program of the Institute: Understanding the regulation of cell replication is of great importance to biologists studying human ageing. With increasing human age there are many examples of delayed or impaired response by tissues and/or cells to normal stimuli for cellular replication (e.g., wound healing, immune response). Therefore, our studies aimed at defining the properties which influence cell replication may provide insight into what agents might be effective in reversing decreased cell replication, at first *in vitro* and perhaps later *in vivo*.

Human chromosomal aneuploidy is associated not only with increased *in vivo* ageing, but also with malignancy. Certain human chromosomal aneuploid disorders, such as Down's syndrome, reveal an increased frequency of neoplasms as well as many *in vivo* and *in vitro* features of accelerated ageing. The *in vitro* study of cellular replication and nucleic acid metabolism in cells derived from these patients should provide pertinent information on the relationship between chromosomal aneuploidy and malignant ability as well as on the cellular pathophysiology of this common human disorder.

Proposed Course: Cell population replication rate, percent replicating cells and cell cycle times will be measured in cell cultures derived from young and old human donors. If these cell replication parameters are altered in cultures derived from old donors, it will establish a more appropriate *in vitro* model for studying cellular ageing. If there is no difference in cell replication between cultures derived from young and old donors, these results would further question the validity of late passage WI-38 cells representing *in vivo* human ageing.

Drug-induced aneuploid cell cultures will be examined to determine if they have the decreased *in vitro* lifespan, decreased cell replication rate and altered nucleic acid metabolism observed in cultured cells from patients with human chromosomal aneuploid disorders.

Viral probes will be utilized to test the fidelity of macromolecular synthesis in skin fibroblast cultures derived from young and old human donors as well as from aneuploid and control normal cell cultures (in collaboration with Dr. Jochen Heine).

Attempts will be made, utilizing growth-promoting and -retarding agents, to examine the interconversion of replicating and non-replicating cells.

RNA metabolic studies will be concluded in WI-38 cells with an analysis of messenger and ribosomal RNA synthesis, degradation and turnover.

Honors and Awards: Dr. Schneider presented part of this work at the Gerontological Society meetings in Miami, Florida on November 7, 1973.

Dr. Schneider presented a seminar related to this work at the University of Pennsylvania, Department of Genetics, Philadelphia, Pennsylvania on January 9, 1974.

Dr. Schneider presented a seminar related to this work at Johns Hopkins University, Department of Medicine, Baltimore, Maryland on December 12, 1973.

Publications: None

Serial No.: HD-CCP-20
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Effects of the Agouti Locus on Development and Aging in Mice

Previous Serial Number: Same

Principal Investigator: Gary R. Dunn

Other Investigator: George Roth

Cooperating Units: None

Man Years:

Total:	0.1
Professional:	0.1
Other:	0.0

Project Description:

Objectives: An understanding of the genetic regulatory mechansims operating in cells is of basic importance for our understanding of age-related decline in cell function, especially of genes which are expressed throughout the life span of an organism. Our model system is the agouti locus of the mouse and specifically the yellow-agouti allele, A^y. Homozygotes for this particular allele die as early embryos while heterozygotes lack black pigment in their hair, are obese, and have a shortened live span relative to their normally pigmented littermates. Our research has centered on obtaining a fuller understanding of the homozygous lethality of A^y.

Methods Employed: The observation that many organs are affected and adrenal hypertrophy occurs in heterozygous A^y mice, suggests an altered response to adrenal hormones. Fatty acid oxidation of liver has been shown to be inhibited by cortisol. Therefore the fatty acid oxidation of liver homogenates was determined by the release of $C^{14}O_2$ from palmitic acid.

Major Findings: Changes were found in the fatty acid oxidation of both the basal and cortisone treated A^y heterozygotes relative to A^y homozygotes. However, these changes did not appear to be of sufficient magnitude to account for the large physiological differences among the groups.

Significance to Biomedical Research and the Program of the Institute: The complexity of the A^y heterozygote phenotype suggests an underlying hormonal defect. However, neither our studies on response to hormone nor other work on hormone levels has revealed a probable basis for the A^y syndrome.

Proposed Course: This project has been terminated.

Honors and Awards: None

Publications: None

Serial No.: HD-CCP-21
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Use of Dietary Probes to Study Mechanisms of Age Related Immunological and Biochemical Functions (formerly entitled Effect of Dietary Manipulations on Life Span, Incidence of Diseases and Immunologic and Related Functions)

Previous Serial Number: Same

Principal Investigators: C. H. Barrows, Jr.
T. Makinodan

Other Investigator: G. R. Dunn

Cooperating Units: None

Man Years:

Total:	3.0
Professional:	1.7
Others:	1.3

Project Description:

Objectives: Levels of dietary protein intakes ingested at specific intervals of the life cycle which will optimize physiological performance are unknown. Perinatal undernutrition results in certain irreversible changes which markedly affect an organism throughout its life. Dietary protein restriction imposed immediately following weaning results in an increased life span in rodents with average life expectancies. However, little information is available as to whether this latter effect can be brought about in rodents when the dietary restriction is imposed during adulthood or in animals whose life expectancies are dependent upon specific diseases. More important is that the cellular mechanism responsible for these two observations are not known. Therefore, attempts are presently being made using diet as a probe to determine the interrelationships among the effects of varying the dietary protein levels at various stages in the life cycle on longevity, specific immunological and biochemical mechanisms.

Methods Employed:

1) Animals - Approximately 750 BALB/c ♂ animals have been received from NIH and divided equally among the following dietary regimens: a) 24% protein fed throughout the life span; b) 8% protein fed throughout the life span; c) 4% protein fed throughout the life span; d) 24% protein fed until 18 weeks of age (essentially constant body weight from 30 to 32 gr. had been maintained since approximately 14

whether D-fructose-1, 6-diphosphate (FDP) or D-fructose-1-phosphate (FIP) were employed as substrates. The activity of aldolase in muscle did not decrease markedly with age (13%).

The activity of these enzymes were also determined in 4 month old BALB/c ♂ mice fed either a 24,8, or 4% casein diet. A moderate decrease in the catalase activity of liver was observed in mice fed the 8 and 4% as compared to the 24% protein diet (25%). On the other hand, the activity of this enzyme in kidney was unaltered. The aldolase activity in the livers of animals fed the 8 and 4% protein diets were 56 and 103% higher, respectively, than those fed the 24% diet. These increments were essentially the same whether FDP or FIP were employed as substrates. Only a slight increase (13%) in the activity of aldolase in muscle was observed with decreasing dietary protein.

Approximately a 300-fold increase in the specific activity of catalase was achieved during the purification of this enzyme. The ultraviolet and visible spectra of the final preparation were determined in the Cary spectrophotometer and found to be essentially the same as that reported by Price. However, in determining the degree of purity, the ratio of the absorption at 470 mμ to that at 276 mμ was found to be 0.73. This does not compare favorably with the ratio of 1.08 reported by Price in his best preparations (97-98% pure). In addition, in this preliminary study, the recovery was only approximately 50% of that reported by these authors.

At present only the NaCl gradient elution of the purification of aldolase has been completed. A ten-fold increase in the specific activity of this enzyme has been achieved by this first elution. This is greater than the 2.5-fold increase reported by Gershon and is essentially equal to that reported by Gracy.

2) Immunological Studies - The data indicated no effect of diet on the hemolysin and agglutinin titers following primary immunization either at 1 week or 1 month of age with SRBC. However, there was a tendency for the peak values to occur somewhat later (3 days) in the animals challenged at 1 month of age which may suggest that the rate of generation of the antibody-forming cells may have been adversely affected by diets. A decrease, associated with decreasing dietary protein, was found in the hemolysin and agglutinin titers following secondary immunization. This trend suggests that retention of immunological memory can be affected by protein undernutrition. It should be emphasized however that these preliminary experiments only suggest that dietary protein inanition has a marked impact on the immunological responsiveness of animals.

3) Perinatal Undernutrition Studies - The mortality was 95,50 and 10% in the young of mothers who were fed 4,8 and 24% dietary protein levels respectively. A 29% reduction in body weight was observed in the young of mothers fed 8% dietary protein. A similar reduction in the weights of the livers, kidneys, hearts and spleens was found so that the organ to body weight ratios were the same in both groups of

weeks of age) at which time the animals were offered 8% diet; and e) 24% protein fed until 18 weeks of age at which time the animals were offered 4% diet. Mortality statistics will be determined on these mice and animals will be sacrificed at 2,5,12,18 and 24 months of age for immunological and biochemical studies.

An additional 170 BALB/c ♂ mice have been received for preliminary immunological studies and divided equally among dietary regimens a), b), and c). Sixty animals, equally divided among the diets, were immunized with sheep red blood cells (SRBC) either at 1 week or 1 month of age. They were bled on days 0,3,6,9,12 and 15. The animals were reimmunized at 20-21 weeks of age.

Fifty ♀ and twenty ♂ BALB/c mice, 21 days of age, were multiple housed and placed on 24% protein diet. At seven weeks of age, they were mated. The pregnant females were isolated and continued to be fed the 24% protein diet until birth, at which time the mothers were offered 24,18,12,8 or 4% protein diets.

2) Biochemical Studies - Two enzymes were chosen, viz. aldolase and catalase, on the basis that they can be quantitatively estimated immunologically as well as enzymatically and rates of synthesis can be estimated by the use of irreversible inhibitors or employing radioactive isotopes. Aldolase was determined by Gershon's modification (Proc. Nat. Acad. Sci.: 70, 909-913, 1973) of Gracy's method (Arch. Biochem. Biophys.: 136, 480-489, 1970). Catalase was determined by Samis' modification (Gerontologia: 17, 105-115, 1971) of the method of Rechcigl, et al. (Cancer Res.: 22, 874-880, 1962). The purification of aldosase was attempted by the method of Gracy, et al. (JBC: 244, 3913, 1969). This method is based on column chromatography employing a NaCl gradient, followed by a substrate (FDP) elution. The purification catalase was attempted by the method of Price, et al. (JBC: 237, 3468, 1962). This method is based essentially on salt precipitations and dialyses.

3) Immunological Studies - Hemolysin and agglutinin titers were carried out by the standard microtitration method.

4) Perinatal Undernutrition - The young of mothers fed the various diets were weaned at 21 days of age and sacrificed. The organ to body weight ratios were determined. The total number and the number of viable cells were estimated.

Major Findings:

1) Biochemical Studies - In order to determine whether aldolase and catalase were influenced by age and diet, two studies were carried out. In the first, these enzymes were determined in ♂ BC_3F_1, mice, 3 months and 31 months of age. The data demonstrated a marked decrease with age on the activity of catalase in kidney (58%) with a smaller decrement in liver (17%). A decrease in the activity of aldolase with age was observed in liver (35%). This decrement was essentially the same

animals. However the reduction in thymic weight (14.5%) was not comparable to the reduction in body weight so that the thymic to body weight ratio was significantly increased in the program of mothers fed the low dietary protein observed. The lack of a significant difference in the number of cells or viable cells (trypan blue exclusion) either per unit wet weight or per total organ must be tempered with the increased variability which occurs in cell counts as compared to the measurement of the wet weight of organs.

Significance to Biomedical Research and the Program of the Institute:

These dietary manipulations may retard the onset and consequences of immunodeficiency diseases of aging. As such, we may have a better insight into the mechanisms of regulation of immune activities.

Proposed Course:

Purification of aldolase and catalase will be continued until adequate preparations for the immunization of rabbits can be carried out. The poor yields of catalase may be accounted for by the fact that recent studies indicate that the enzyme is contained within microbodies or peroxisomes and the use of non-ionic detergents such as Triton x-100 may increase the yields. Efforts will then be made to insure production of monospecific antibody reagents.

Additional immunological studies must be carried out kinetically to determine whether the effects of diet observed following re-immunization is due to the loss of memory cells or emergencies of suppressor cells in animals fed the low dietary proteins. The other alternative is the effect of the additional length of time the animals were maintained on the diets. On the basis of the validity of the observations that diet does affect the humoral immune activities, the regulation of cell-mediated immune activities will be carried out.

Efforts will be made to determine whether the deficits in organ and body weights observed in the young of mothers fed the 8% casein diet are reversible by feeding the young the 24% dietary protein following weaning. In addition, efforts will be made to determine the effects of various dietary protein levels offered mothers during gestation as well as lactation. These studies will include the measurement of biochemical variables such as enzyme activities and the concentrations of DNA, RNA, and protein of a number of organs.

Honors and Awards: None

Publications: Silbert, J.A. and Barrows, C.H., Jr.: Cellular senescence and death. In Dikstein, S. (ed.): Fundamentals of Cell Pharmacology. Springfield, Ill., Charles C. Thomas, 1973, pp. 471-489.

Serial No.: HD-CCP-22
1. Gerontology Research Center
2. Laboratory of Cellular and
Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Ageing and Genetically Defective Mammalian Skeletogenic Cells and Connective Tissues

Previous Serial Number: Same

Principal Investigator: Dorothy F. Travis

Other Investigators: None

Cooperating Units: Laboratory of Biochemistry
National Institute of Dental Research
Bethesda, Maryland

Project Description: This project has been discontinued.

Serial No.: HD-CCP-23
1. Gerontology Research Center
2. Laboratory of Cellular and
Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Low Temperature Effects on Cells of Ageing Individuals (formerly entitled "Studies on the Comparative Physiology of Ageing and Environmental Effects on the Ageing Process")

Previous Serial Number: Formerly HD-AG-37

Principal Investigator: Mary Anne Brock

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1.0
Professional: 1.0
Others: 0.0

Project Description:

Objectives: To define the age-related functional and structural characteristics of pre- and post-mitotic cell types, and specifically 1) to investigate the possible age-related differences in lymphohemopoietic cells, particularly biomembrane systems, reflected in differential susceptibility of the cells to freezing damage and in their ability to recover after thawing; and 2) to establish freezing procedures suitable for long-term storage of subpopulations of lymphocytes.

Methods Employed: Mouse splenic cell suspensions were subjected to controlled rate freezing with the Linde BF-4 Biological Freezing System and thawed. Functional capacity of the frozen-thawed cells was assessed by H^3-thymidine incorporation in vitro with and without stimulation by the T-cell mitogens, PHA and SEB, and the B-cell mitogen, LPS.

Major Findings: Studies on controlled rate freezing were initiated which included 1) organizing a laboratory for freezing experiments, 2) thoroughly testing the controlled rate cooling equipment currently available, and 3) developing procedures for the controlled rate freezing of lymphocytes and the assessment of their viability after thawing. These latter procedures incorporated published controlled rate cooling protocols and in vitro assays currently used in this laboratory, and results indicate that the equipment now in use is only suitable for routine, non-critical controlled rate freezing.

Preliminary experiments showed that under optimum conditions full recovery of frozen-thawed splenic lymphocytes was possible. More commonly, however, the number of cells recovered following any experimental manipulation was reduced to 60% when compared with untreated controls. Whether this reduction depends on current methods is being investigated. The number of cells recovered was comparable in lymphocytic suspension in RPMI-1640 with 10% fetal calf serum and with 10% DMSO in some samples whether the suspensions were 1) held at 4°C in medium plus fetal calf serum for 4 hrs., 2) held at 4°C in medium, fetal calf serum plus DMSO for 4 hrs., or 3) frozen at -1°C/min. and thawed in medium, fetal calf serum plus DMSO, this procedure required 4 hrs.

A differential loss in activity of a subpopulation of lymphocytes that are responsive to PHA, a T-cell population, was observed in all of the experimental groups when compared to fresh, control cells.

To resolve whether age-related differences can be detected in the freezing properties of lymphohemopoietic cells will depend on an instrument with a range of freezing rates ten times slower than that presently available and without the variability in cooling rate introduced at the point of freezing. A new instrument developed by the NIH Biomedical Engineering and Instrument Branch should meet these requirements, and accordingly has been ordered.

Significance to Biomedical Research and the Program of the Institute: The decline in the functional capacity of the cellular components of the immune system with age may result from intrinsic changes or defects in the various cell types of the lymphohemopoietic system. The response of different cell types to the stress of freeze-thaw in animals of different ages can reflect alterations, particularly those associated with biomembrane systems. Furthermore, controlled rate cooling techniques may be used to separate subpopulations of lymphocytes for further study at the cellular level.

Proposed Course: Major emphasis will be placed on the definition of possible age-related changes in the biomembrane systems of mammalian lymphoid cells using controlled rate cooling techniques to assess cellular resistance to stress. Age-related changes in plasma membranes of lymphocyte subpopulations will be assessed kinetically with transmission and scanning electron microscopy.

Honors and Awards: Dr. Brock served as a member of the Board of Governors of the Society for Cryobiology.

Dr. Brock was an invited speaker at the 1974 AAAS Annual Meeting in the symposium, Annual Biological Clocks; Circannual Clocks, held in San Francisco, February 1974.

Dr. Brock was invited to chair a session for the AAAS Symposium, Annual Biological Clocks; Circannual Clocks, held in San Francisco, February 1974.

Dr. Brock was invited to the Fifth International Interdisciplinary Cycle Research Symposium, Noordwijk, The Netherlands, to be held June 1974.

Dr. Brock was elected a Fellow of the Gerontological Society.

Publications: Brock, M.A.: Growth, developmental and longevity rhythms in *Campanularia flexuosa*. *Amer. Zool.*, in press.

Brock, M.A.: Circannual rhythms I. Free-running rhythms in growth and development of the marine cnidarian, *Campanularia flexuosa*. *Comp. Biochem. Physiol.*, in press.

Brock, M.A.: Circannual rhythms II. Temperature-compensated free-running rhythms in growth and development of the marine cnidarian, *Campanularia flexuosa*. *Comp. Biochem. Physiol.*, in press.

Brock, M.A.: Circannual rhythms III. Rhythmicity in the longevity of hydranths of the marine cnidarian, *Campanularia flexuosa*. *Comp. Biochem. Physiol.*, in press.

Serial No.: HD-CCP-24
1. Gerontology Research Center
2. Laboratory of Cellular and
   Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Effects of Aging on the Modulation of Proliferation and Differentiation of Immunocompetent Cells (formerly the following two projects--Characterization of Age-Related Defect in the Proliferative Capacity of Lymphocytes and Characterization of Cellular Deficiencies in Age-Related Decline of the Humoral Immune System)

Previous Serial Numbers: HD-CCP-12 and HD-CCP-13

Principal Investigators: Katsuiku Hirokawa
T. Makinodan

Other Investigator: Jochen Heine (9/16/73 EOD)

Cooperating Units: Oak Ridge National Laboratory
Oak Ridge, Tennessee

University of Nebraska
Omaha, Nebraska

Man Years:

Total:	4.3
Professional:	1.6
Others:	2.7

Project Description:

Objectives: Immunologic activities generally decline as individuals age. This is due primarily to the decline in the proliferative and differentiative capacities of antigen-sensitive precursor cells caused by (a) a decline in their functional potential through somatic mutation, (b) an increase in the proportion of regulator cells with suppressive activity, (c) a decrease in the proportion of regulator cells with enhancing activity, and/or (d) a decrease in the concentration of nutritional factor(s). The objective of this project is to determine the cellular and molecular mechanisms responsible for the decline in functional immune activities. Currently we are devoting our efforts to the nature of age-related cellular changes.

Methods Employed: Splenic cells from young and old mice were separated into subpopulations by their differences in density, adherence to glass beads and susceptibility to various antiserum reagents. The subpopulations were then assessed for their functional properties, as judged by their ability to respond to specific mitogens and antigens in vitro. In addition, the T cell-transforming capacity of thymic epithelial tissue was assessed by implanting thymic fragments of various ages into T cell-deficient young mice, i.e., young mice which had been previously thymectomized, lethally x-irradiated and reconstituted with bone marrow cells. Assays for functional T cells at various intervals thereafter were carried out.

Major Findings: 1. There is no appreciable difference in the number and proportion of the major cell types (T, B, and A cells) in the spleen of young and disease-free, old, long-lived mice. However, the proportion of the less dense T-mitogen unresponsive cells is increased at the expense of the denser T-mitogen responsive cells. This would suggest that the less dense cells, possibly reflective of a shift in the nuclear-cytoplasm ratio, are being selected with age.

2. The DNA synthetic activity of mitogen stimulated splenic T cells declines with age, but not that of mitogen stimulated spleen B cells, suggesting an unbalanced shift with age in cell-mediated, T-dependent humoral and T-independent humoral immune functions.

3. The decline with age in DNA synthetic activity of T cells is correlated most strikingly with the level of cyclic-GMP. Because maximum changes in the level of cyclic nucleotides occur within minutes after mitogen stimulation and because there is evidence that changes in the intracellular levels of the cyclic nucleotides initiate and regulate cellular proliferation and differentiation, these results suggest that profound changes are occurring at the membrane level with age.

4. The thymic epithelial tissue of old mice, in contrast to that of young mice, when implanted into T cell-deficient young mice appears to be incapable of transforming precursor cells into functional T cells. The loss in the functional capacity with age of epithelial cells can be correlated morphologically with the appearance of bizarre mitochondria, whirled membranous materials and cystic dilation of secretory vacuoles, and a decrease in the mass of the Golgi apparatus and the number of free ribosomes. Whether or not this age-related defect is irreversible or reversible remains to be resolved.

Significance to Biomedical Research and the Program of the Institute: The decline of functional immune activities with age has an obvious effect upon general senescence and extension of health. If the cell types and regulator factors which are responsible for the decline can be identified and characterized, this will be a significant step toward determining the cause for the decline and the approaches to delay and reverse the decline. Moreover, a comprehensive understanding of the effects of aging affects other vital tissue and organ systems, especially those involving cells undergoing proliferation and differentiation.

Proposed Course: Further studies will continue to separate, identify and characterize the cell types responsible for the decline in functional immune activities and relate functional changes to ultrastructural changes. Studies will also be initiated to characterize the factor(s) which can modulate the proliferation and differentiation processes.

Honors and Awards:

Dr. Makinodan was invited to teach a two-week course in cellular immunology and aging at the 1973 Summer Institute for Study in Gerontology at the Ethel Percy Andrus Gerontology Center, University of Southern California, Los Angeles, California, July 16-27, 1973.

Dr. Makinodan was invited to lecture in the Annual Biology of Aging Course, sponsored by ADAB, NICHD, held at the University of Minnesota, Duluth, Minnesota, August 13-17, 1973.

Dr. Makinodan was invited to participate in a lecture series of the Microbiology Course, Department of Microbiology, Johns Hopkins University, Baltimore, Md., September 17, 1973.

Dr. Makinodan was invited to give a lecture at the International Research Workshop on the Biology of Aging, held at the Ciba Foundation, London, England, October 22-24, 1973 and sponsored by the National Institute for Medical Research, London, England.

Dr. Makinodan was invited to give a lecture at the Third Brazilian Congress of Geriatrics and Gerontology held in Rio de Janeiro, Brazil, November 4-6, 1973.

Dr. Makinodan served as Co-Chairman of the Session on Immunology, Biological Sciences Section of the Gerontological Society at the 26th Annual Meeting held in Miami Beach, Florida, November 5-9, 1973.

Dr. Makinodan was appointed as chairman-elect of the Biological Sciences Section of the Gerontological Society.

Dr. Makinodan was invited to give a lecture at the seminar on the Sciences and Aging sponsored by the Council on Aging, University of Kentucky, Lexington, Kentucky, November 17, 1973.

Dr. Makinodan was invited to attend the Symposium on Molecular and Cellular Mechanisms of Aging sponsored by the French Medical Research Council, Paris France, December 5-7, 1973 and to give a lecture entitled "Cellular Basis of Immunosenescence."

Dr. Makinodan was invited to give a seminar by the National Institute for Medical Research, London, England, December 10, 1973.

Dr. Makinodan was invited to give two seminar lectures in the Department of Zoology, Oxford University, Oxford, Ohio on January 24, 1974.

Dr. Makinodan was invited to give a lecture in the Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts on February 11, 1974.

Dr. Makinodan was invited to give a lecture by the Harvard Medical Society, Massachusetts General Hospital, Boston, Massachusetts on February 12, 1974.

Dr. Makinodan was invited to attend and give a lecture at the meeting entitled "Interaction of Radiation and Host Immune Mechanisms of Malignancy" held March 23-27, 1974 at White Sulphur Springs, West Virginia, and sponsored by the National Cancer Institute, Committee for Radiation Therapy Studies, the Radiation Study Section and the Subcommittee on Radiobiology of the National Academy of Sciences.

Dr. Makinodan was invited to participate and lecture in a symposium on "Biomedical Aspects of Aging" in the 140th Annual Meeting of AAAS, held in San Francisco, California, February 25-March 2, 1974.

Dr. Makinodan was invited to give a lecture in the Symposium on Development and Aging in Organ Systems at the meeting of the Federation of American Societies for Experimental Biology held in Atlantic City, New Jersey, April 11, 1974.

Dr. Makinodan served as Associate Editor of the following journals: Journal of Cellular Physiology, Blood, Mechanisms in Aging and Development, and Journal of Immunology.

Dr. Makinodan served as AAI representative to the FASEB Public Information Committee.

Publications:

Heidrick, M.L. and Makinodan, T.: Nature of cellular deficiencies in age-related decline of the immune system. Gerontologia 18: 305-320, 1972.

Quinn, R.P., Price, G.B., Ellis, J.M., and Makinodan, T.: Catabolic half-lives of immunoglobulin and albumin as a function of age in mice. J. Gerontol. 28: 257-264, 1973.

Price, G.B. and Makinodan, T.: Aging: Alteration of DNA-protein information. Gerontologia 19: 58-70, 1973.

Heidrick, M.L. and Makinodan, T.: Presence of impairment of humoral immunity in nonadherent spleen cells of old mice. J. Immunol. 111: 1502-1506, 1973.

Makinodan, T.: Future biologic research on aging. In: Proceedings of the 4th Seminar: The Sciences and Aging. Lexington, Ky., Counc. on Aging, Univ. of Kentucky, November 16, 1973, pp. 1-12.

Halsall, M.K. and Makinodan, T.: Analysis of the limiting dilution assay used for estimating frequencies of immunocompetent units (IU). Cell. Immunol. 11: 456-465, 1974.

Makinodan, T., Heidrick, M.L., and Nordin, A.A.: Immunodeficiency and autoimmunity in aging. In: *Second International Workshop on Primary* Immunodeficiencies in Man, February 4-8, 1973. Stanford, Conn., Sinauer Press (in press).

Nordin, A.A. and Makinodan, T.: Humoral immunity in aging. *Fed. Proc.* (in press).

Makinodan, T.: Cellular basis of immunosenescence. In: *Symposium on the Molecular & Cellular Mechanisms of Aging, December 5-7, 1973, Paris, France*. (In press).

Makinodan, T. and Adler, W.H.: Cellular aspects of age-related decline in immune activities. *Fed. Proc.* (in press).

Goodman, S.A. and Makinodan, T.: Effect of age on cell-mediated immunity in long-lived mice. *Clin. Exp. Immunol.* (in press).

Vazquez, J. and Makinodan, T.: Aging and the immune system. A brief summary of current knowledge. In: *Epidemiology of Aging*, (in press).

Serial No.: HD-CCP-25
1. Gerontology Research Center
2. Laboratory of Cellular and
   Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Role of Cell Membrane Structures in Cellular Recognition Phenomena

Previous Serial Number: None

Principal Investigator: W. H. Adler (9/15/73 EOD)

Other Investigators: J. W. Heine (9/16/73 EOD)
G. Stoltzner (11/1/73 EOD)

Cooperating Units: None

Man Years:

Total:	2.6
Professional:	2.0
Other:	0.6

Project Description:

Objectives: Lymphocytic cells have been subclassified according to membrane structure, mechanical characteristics and functional criteria in several different assay procedures. However, correlation of the different subclassifications in terms of surface markers, separation techniques, and function has not been studied in terms of changes in subpopulation proportions or in terms of individual cellular differentiation changes and the effects of these changes on overall immune function *in vivo*.

Methods Employed: The basis of much of the functional assay will be an *in vitro* lymphocyte culture system in which proliferative capacity will be measured. This system will also be useful in determining cellular interaction and the role of soluble factors in augmenting specific cell function.

Soluble membrane preparations, using detergent or high molar salt elution will be used to determine both the role of membrane components in recognition of antigen and the antigenic nature of the components themselves.

Cell Separation techniques are being perfected that should allow up to 99% pure subpopulations of lymphocytes to be obtained and classified as to both type and function.

Membrane interaction with defined and tagged mitogens will be possible using various microscopic analysis and using various physical separation techniques.

Major Findings: Subpopulations of spleen cell lymphocytes can be shown to have markedly different sensitivity to x-irradiation and to mitomycin.

PHA, and LPS responsive subpopulations are more sensitive (2.5x) to the proliferative inhibiting effects of x-irradiation than are the Con-A, Enterotoxin, or MLC responsive subsets.

The LPS responsive subset is much more sensitive to the inhibitory effects of mitomycin-c (4x) than are any of the other subsets of spleen lymphocytes.

The in-vitro micro-culture of mouse lymphocytes is an economical, simple, time saving, and reproducible assay of cellular function.

Cell membrane soluble antigens are able to inhibit antibody killing of target cells, but are ineffective in the inhibition of cell mediated killing of target cells. The soluble antigen is minimally stimulatory on allogeneic lymphocytes, can not inhibit an MLC reaction, but is able to inhibit the generation of cytotoxic effector cells by the MLC reaction. The soluble antigen is a poor immunogen, both in stimulating the appearance of antibody but especially it is ineffective in stimulating the appearance of immune MLC reactive cells and cytotoxic effector cells. (It will not stimulate the appearance of effector cells.)

A subpopulation of thymus cells, which are LPS responsive can be influenced to appear by incubation of the thymus cells with the T-cell mitogen- Con-A. This finding is being investigated in terms of cell interaction, cell differentiation, and the role of cellular mediators in cell function.

The role of LPS on "B" cell function in several in vitro cell culture systems is being explored after the very significant finding that about 30% of commercially available fetal calf serum is endotoxin contaminated.

Significance to Biomedical Research and the Program of the Institute:

Without a method of classifying individual lymphocytes, separating them and obtaining homogeneous populations, and then examining the functional properties of these populations, an understanding of immune function, cellular differentiation and changes in function can only be superficial. The correlation of the cell surface structures, membrane properties and function will help in understanding the changes seen in the immune system in the intact animal.

Proposed Course: Since this project is only in the initiation phase, the investigations proposed are still to be examined in detail. Progress so far has been extremely encouraging, the techniques which have been developed are yet to be fully exploited.

Honors and Awards:

Dr. Adler was invited to give a seminar on "In Vitro Lymphocyte Reactions" at the Department of Pathology, University of Florida, Gainesville, Florida on February 5, 1974.

Dr. Adler was invited to participate in a seminar on "Theoretical Aspects of Aging" and present a paper on "An Autoimmune Theory of Aging" at the University of Miami, Miami, Florida, February 6-7, 1974.

Dr. Adler was invited to give a seminar on "Mixed Lymphocyte Cultures" at the Carnegie Institute, Baltimore, Maryland, February 11, 1974.

Dr. Adler served as chairman of the "Cellular Immunology" session at the Annual AAI Meetings in Atlantic City, April 9, 1974.

Dr. Adler has been asked to serve as co-chairman of the "Immunology of Aging" session at the International Congress of Immunology, Brighton, England, July 28, 1974.

Publications:

Adler, W.H.: An autoimmune theory of aging. In Rockstein, M. (ed.): Theoretical Aspects of Aging. Academic Press, New York, New York. In press.

Serial No.: HD-CCP-26
1. Gerontology Research Center
2. Laboratory of Cellular and
Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mechanisms of the Maternal Age Effect as Expressed by an Increased Frequency of Chromosomal Aneuploid Offspring with Increased Maternal Age

Previous Serial Number: None

Principal Investigators: Gary Dunn
Edward L. Schneider

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.0
Professional:	0.8
Other:	0.2

Project Description:

Objectives: Epidemiological studies have clearly demonstrated that the risk of having offspring with chromosomal aneuploidy increases dramatically with increasing human maternal age. Although there has been considerable speculation about the etiology of this maternal age effect, direct experimentation upon humans to study this effect is not feasible. Thus there is an urgent need for the creation of an animal model. The mouse was chosen as an animal model because of its rapid breeding rate, large number of offspring, genetically defined inbred strains, and the availability of retired female breeders for comparison with young females. Initially, the methodology for obtaining an adequate number of well spread chromosome metaphase preparations will be worked out. Subsequently, a survey of chromosomal aneuploidy in retired breeders from various inbred mouse strains should reveal the strain with the highest frequency of chromosomal aneuploid offspring. This strain will then be used to determine the effects of intrinsic (oocyte) and extrinsic (uterine, hormonal, etc.) factors on the maternal age effect. In addition, embryo maturation and implantation will be concurrently examined as a function of maternal age.

Methods Employed: Retired breeders and young females from six different strains are mated with young males of the same strain. These strains were selected for their radiosensitivity (SJL/J), immunological dysfunction

(NZB, A/J), premature reproductive decline (A/J), unusual oocyte depletion with age (CBA/J) and general availability (C57Bl/6; C3HeB/FeJ). Pregnant females are sacrificed between nine and eighteen days gestation and the number of pre- and post-implantation dead and living embryos are determined. Chromosome preparations are made from all living embryos. The number of embryos, frequency of pre-implantation and post-implantation death, and frequency of chromosome preparations with more or less than a diploid chromosome complement are calculated.

Major Findings: Preliminary results suggest that retired mouse breeders have significant numbers of chromosomal aneuploid embryos, indicating that the mouse may be an appropriate animal model for the maternal age effect. In addition, the autoimmune-prone NZB strain was noted to have a significant increase in the frequency of pre-implantation death.

Significance to Biomedical Research and the Program of the Institute: Human chromosomal aneuploid disorders are extraordinarily common as illustrated by Down's syndrome or mongolism (Trisomy for chromosome 21) having a frequency of 1:600 live births. At the maternal age of 45, the frequency of Down's syndrome rises to approximately 1:40. Even with the advent of prenatal diagnosis, children with this disorder will continue to be born and require a lifetime (mean lifespan ~ 30) of specialized care.

Proposed Course: The strain survey should determine the mouse inbred strains with the highest frequency of age related chromosomal aneuploidy and pre-post-implantation death. Further studies will be directed at delineating the role of proposed etiologic factors including radiation, autoimmunity, infectious agents, and uterine environment.

Honors and Awards: None

Publications: None

Serial No.: HC-CCP-27
1. Gerontology Research Center
2. Laboratory of Cellular and
   Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Characterization of the Immune System of Aging Mice with Immunodeficiency Diseases

Previous Serial Number: None

Principal Investigator: Toshio Hirano

Other Investigators: T. Makinodan
Albert A. Nordin

Cooperating Units: None

Man Years:

Total:	1.3
Professional:	1.2
Others:	0.1

Project Description:

Objectives: The goal of this project is to characterize the immune system in aging mice of various genetic strains that show a high frequency of immunodeficiency disease. The relationship between the immunological disorders and the regulation, in the form of suppression, of the immune system will be investigated.

Methods Employed: 1) A modification of the spleen cell culture system of Mishell and Dutton is used. Spleen cells are cultured with mitomycin-C treated allogeneic spleen cells or heterologous erythrocytes at 37°C for 5 days.

2) Cytotoxicity assay - ^{51}Cr labeled EL-4 cells which are derived from C57Bl/6 mice are mixed with cultured NZB spleen cells and incubated for 8 hours. After incubation, cold PBS is added, the cultures centrifuged and the radioactivity of the supernatant counted.

3) Plaque-forming cell assay - routine technique used to detect IgM and IgG antibody-producing cells.

4) Direct Coombs test is a routine method.

Major Findings: 1) Old male NZB mice - Cell-mediated cytotoxicity was decreased in all aged mice (10-20 months old).

2) Old female NZB mice - There were three different types of responsiveness observed in these animals: 1) cell-mediated immunity (CMI) was normal, 2) CMI was higher than normal and 3) CMI was markedly depressed. None of these types of responsiveness correlated with the autoimmune disease as judged by the Coombs test.

3) Optimum antigen dose is the same for each mouse irrespective of the disease state.

Significance to Biomedical Research and the Program of the Institute: This proposal offers three main significant contributions: 1) the mechanism of the development of autoimmune disease, 2) the mechanism of the immune response by characterizing the abnormal regulation of diseased NZB mice, and 3) the abnormal regulation in aged NZB mice may be a suitable model to test the theoretical consideration that autoimmunity is a critical problem in immunosenescence.

Proposed Course: 1) The relationship between the abnormal immune response and the autoimmune disease will be characterized.

2) The relationship between ageing and autoimmune disease will be investigated.

Honors and Awards: None

Publications: None

Serial No.: HD-CCP-28
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Conscription and Polyadenylation of Genetic Messages During Early Embryogenesis

Previous Serial Number: None

Principal Investigator: D. W. Slater

Other Investigator: D. H. Gillespie

Cooperating Units: Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health

Man Years:

Total:	2.0
Professional:	1.0
Other:	1.0

Project Description:

Objectives: To determine the mechansims involved in the processing and selective recruitment of genetic messages during early development.

Methods Employed: The procedures utilized for the preparation and characterization of genetic messages native to pertinent subcellular fractions from eggs and embryos have been described in the publications cited below. For a detailed description of the RNA-RNA molecular hybridization procedure employed the reader is referred to Slater, et al. [Nature, 240 (1972) 333-337].

Major Findings: Tracts of polyadenylic acid [poly(A)] are a proprietary property of most eukaryotic genetic messages. While such poly(A) tracts thereby provide a unique tool with which to identify and characterize genetic messages, the biologic function of these poly(A) sequences is presently unknown. As the poly(A) segments are not translated, they are currently presumed to have a regulatory role in message metabolism.

We had previously established via molecular hybridization between unlabeled RNA prepared from sea urchin eggs, zygotes or early embryos and [^{3}H] poly(U) that fertilization results in a 2.5-fold net synthesis of poly(A).

This net synthesis occurs in the period between Insemination and the two-cell stage [Slater, *et al*., *Nature*, 240 (1972) 333-337]. Subsequent analysis of the poly(A) content of egg and embryo revealed that this massive synthesis of poly(A) is a phenomenon unique to the initial inception of embryogenesis as it is not again encountered at any other developmental stage examined to date through gastrulation.

Post-fertilization polyadenylation is not quantitatively altered by such inhibitors of DNA-directed RNA synthesis as ethidium bromide or actinomycin D. Examination of the size distribution of the messenger RNA populations adenylated in the presence of ethidium bromide or actinomycin D now permits us to further state that the immunity to these antimetabolites is also reflected in a qualitative coincidence between adenylated RNAs present in embryos subjected to transcriptive inhibition and those from appropriate untreated controls (Slater, *et al*. in "Control of Post-fertilization Adenylation", in manuscript).

In contrast to the lack of any obvious transcriptive requirement, early embryogenesis is decidedly contingent on continuous protein synthesis. Inhibition of the vigorous increment in translation which normally follows fertilization does not, however, inhibit post-fertilization polyadenylation. Indeed, suppression of post-fertilization protein synthesis, which results in the total and irreversible arrest of development, not only failed to suppress polyadenylation, but, suprisingly, significantly stimulated the amount of poly(A) synthesized. Thus, post-fertilization poly(A) synthesis actually appears to be restricted by some translation-dependent activity which attends fertilization. Examination of the size distribution of genetic messages adenylated in the absence of protein synthesis now indicates, however, that species of RNA adenylated during translational suppression differ from those which serve as primers in either untreated controls or transcriptively suppressed embryos (Slater, *et al*. in "Control of Post-fertilization Adenylation", in manuscript). This finding implies that post-fertilization protein synthesis is critical not only to the quantitative regulation of polyadenylation but, moreover, may be prerequisite to the selection of the specific genetic messages normally adenylated.

Post-transcriptive polyadenylation in adult somatic cells is believed to occur prior to transport from the nucleus into the cytoplasm. Since the inhibition of transcription results in the suppression of the synthesis of prerequisite RNA primers, the arrest of DNA-dependent RNA synthesis by actinomycin D and ethidium bromide should consequently have curtailed the post-fertilization synthesis of poly(A). As noted above, however, this did not prove to be the case in the embryonic material employed for these studies. Given the substantial population of genetic messages with which the unfertilized egg is endowed, the possibility therefore existed that the observed adenylation was supported by pre-existing cytoplasmic genetic messages of maternal origin. Due to the fortunate biology of the system, it was possible to directly assess the validity of this novel contention. Thus, as only one to two nuclei are present per embryo when the initial burst of poly(A) synthesis occurs, nuclear RNA should, it could be predicted, display at some juncture at or prior to the two-cell stage a poly(A) titer far in excess of its cytoplasmic counterpart composed principally of unadenylated ribosomal RNA if the adenylation was intranuclear. Conversely, if the poly(A) tracts are appended to pre-existing primers it follows that under appropriate *in vivo* labeling conditions wherein both newly transcribed genetic messages as well as poly(A) are concomitantly labeled, pre-existing primers of oogenic origin should be evident which contain labeled poly(A) tracts covalently adjuncted to unlabeled RNA primers.

First, with regard to the poly(A) titer of nuclear RNA, we have established that the poly(A) content of nuclear RNA and its comparative contribution to total poly(A) numerically eliminated the possibility that post-fertilization polyadenylation is exclusively or even primarily an intranuclear event. Second, examination via *in vivo* labeling of the molecular morphology of RNA primers adenylated following fertilization by a variety of analytical procedures conclusively indicates that the poly(A) sequences synthesized are indeed appended to pre-existing unlabeled genetic messages of oogenic origin. Hence, if polyadenylation is involved in the initial editing of presumptive genetic messages, this process is not, in embryonic cells, proprietary to the nucleus unless a given codogenic transcript can undergo more than one round of polyadenylation. Alternatively, if polyadenylation serves a dual purpose instrumental to both processing as well as a possible capacitation prerequisite to recruitment of genetic messages into the translational apparatus, the extended lag between the synthesis of oogenic transcripts and their ultimate utilization in the embryo may reflect a unique situation wherein this normally singular event must be separated in time with a resulting secondary cycle of, in this case, cytoplasmic adenylation.

If the nucleus is not the depository of either pre-existing polyadenylated genetic messages or presumptive primers, then they must reside in the cytoplasm. Indeed, the question of where these latent genetic messages are localized in the unfertilized egg has been debated for some six or seven years. By using a subcellular fractionation approach similar to that employed to assess the poly(A) content of nuclear RNA, we have decisively answered this question and established that prior to fertilization the bulk of genetic messages are localized in the subribosomal fraction of the ovum with fertilization subsequently eliciting a rapid transposition of adenylated genetic messages into the ribosomal fraction.

Finally, the unusual size of the adenylated genetic messages present in egg and embryo deserves comment. We have established that $>$ 50% of the adenylated RNA present in the egg and embryo exhibit molecular weights in excess of 1.5×10^6 daltons. While the function of these inordinately high molecular weight cytoplasmic genetic messages is not presently known, findings obtained from both *in vivo* labeling experiments and molecular hybridization preclude ascribing their size to random aggregation. Interestingly, these high molecular weight genetic messages do, however, appear to be a hallmark of embryonic material as they are not found in appreciable quantities in the cytoplasm of normal adult cells.

Significance to Biomedical Research and the Program of the Institute:

In all animals examined to date early embryogenesis appears to be contingent on the expression of latent genetic messages synthesized during oogenesis. Attempts to define the mechanisms governing this selective recruitment of pre-existing genetic information into the translational apparatus logically necessitate the identification of codogenic transcripts prior to their actual utilization as templates for protein synthesis. Since polyadenylation is a post-transcriptive but pretranslational event, it clearly provides a suitable biological label to probe the mechanics of this processive conscription.

While the pertinence of the research described above to the molecular basis of differentiation is therefore self-evident, its further relevance to genetic engineering both with regard to the purposeful manipulation of early development as well as the specific remediation of genetic defects in somatic cells should also be noted. Thus, from the example of the unfertilized egg with its sizeable population of dormant genetic messages which are systematically recruited during early development, it is clear that the presence in the cytoplasm of a genetic message does not a priori connote the immediacy of its translation into protein. It follows that any attempts to exercise considered therapeutic action on the course of development or, for that matter, cellular functions in general via the introduction of pertinent structural genes or even their RNA cognates without prior knowledge of the cytoplasmic processes operative in the selection of the genetic information to be actually utilized as programs for protein synthesis may prove futile.

Further, given the example of Ferrara β-thalassemia, it is likely that many of the hereditary abnormalities previously ascribed to defects in structural genes may actually prove to be lesions at the level of post-transcriptive expression. The existence of such mechanisms governing the selective utilization of genetic messages suggest that the cell possesses yet largely unknown regulatory capabilities which could be potentially employed to enhance the efficiency with which required genetic messages are used. Alternatively, this same battery of mechanisms connotes the existence of intracellular regulatory functions, perhaps analogous to immunological surveillance, capable of discarding or destructing abnormal genetic messages. From this vantage alone, then, the pertinence of investigations involving message mobilization for the suppression and expression of genetic defects or even viral genomes is quite evident.

Proposed Course: This research will be continued using the information obtained to date in conjunction with newly developed molecular methodologies to further probe the mechanisms responsible for the polyadenylation and conscription of genetic messages during early embryogenesis. Further, arrangements have now been tentatively formalized to initiate similar studies with mammalian embryonic material. These arrangements are, however, contingent on the availability of the considerable financial outlay which such research would require.

Honors and Awards: None

Publications:

Slater, I., Gillespie, D., and Slater, D.W.: Cytoplasmic adenylation and processing of maternal RNA. Proc. Nat. Acad. Sci. 70: 406-411, 1973.

Slater, I. and Slater, D.W.: Polyadenylation and transcription following fertilization. Proc. Nat. Acad. Sci. In press.

NICHD Annual Report
July 1, 1973 through June 30, 1974
Gerontology Research Center
Clinical Physiology Branch

The past year marked the end of the seventh 2-year cycle of tests in the Longitudinal Study of Human Aging. The completion of Cycle G was chosen as the appropriate time for a comprehensive summarization of our longitudinal data. Summaries on these computer-based data involve a stage of detailed review for completeness and accuracy by a variety of methods. This essential editing has been a Herculean task but has now been completed for an extensive array of variables: body composition, bone density, B vitamin study, dietary intake, creatinine clearance, activity and attitudes questionnaire, spirometry, strength and work output, tapping test, nerve conduction velocity, reflex time, smoking history, Cornell Medical Index, hearing and vision tests, antipyrine pharmacokinetics, glucose tests, and serum cholesterol and triglyceride concentrations. The increasing prevalence of disease states with age confounds interpretation of physiological age changes. Thus an essential requisite of the comprehensive summary of these data is the classification of subjects on each visit into specific clinical groups. This task has also been completed through Cycle G.

Since it can be anticipated that several of these variables will not be continued into subsequent test cycles, we have also in the past year devoted time to planning future studies on this volunteer population. The following procedures have just been or will soon be added to our study: (1) Echocardiography - a non-invasive technique for evaluation of myocardial function, (2) Sex hormone function (plasma FSH, LH, free and total testosterone levels), (3) Skin fibroblast culture (as a test of the Hayflick phenomenon in man), (4) Post-heparin triglyceride lipase release, (5) Urinary 24-hour sodium excretion (as an index of sodium intake), (6) Retinal photography (age changes have been inadequately studied and also for assessment of a diabetes endpoint), (7) Ethanol infusion study (pharmacokinetics of the drug and cognitive effects as related to age). Several of these new studies involve collaborative efforts with scientists in other Laboratories of NICHD, NHLI, and the Johns Hopkins University Departments of Medicine and Ophthalmology.

Analysis of a "tapping test" in 638 subjects showed interesting age differences. This is a paper-and-pencil test in which subjects are asked to tap at two targets which are varied in their size and in their distance apart. The goals include both speed and accuracy. The strategies of individual subjects vary; some attempt to achieve accuracy at the expense of time, while others work rapidly with some sacrifice of accuracy. While aging is associated with loss of both speed and accuracy, the loss in speed averages about 0.8% per year of life while the loss of accuracy was only 0.3% per year. Thus older participants chose strategies which protected accuracy at the cost of increased time to perform the task.

Because of a striking decrease in pyridoxal phosphate (Vitamin B6) plasma levels with age which we previously demonstrated, an addition to our computer program for analysis of the one-week dietary diaries in our participants was written in order to estimate dietary intake of this vitamin. A decreased intake of this vitamin was indeed found - the oldest subjects averaged only 1.27 mg intake/day as compared to 1.77 mg/day for the youngest subjects. These mean values should be compared to the NRC recommended allowance of 2 mg/ day. In these 303 subjects, the group who were not taking supplementary vitamin preparations, 6% were taking in less than 1 mg/day and 72% were below the recommended allowance. These data are especially interesting since they are derived from an educationally-advantaged group-the majority of the participants are college graduates.

The detailed Activity Questionnaire has been partially analyzed. The data are of interest not only from the standpoint of the role of physical activity in aging and disease development, but also from the standpoint of social factors which influence activity patterns. Data are being analyzed for total caloric expenditure and also for intensity of expenditure. Thus activities which require at least 0.06 cal/kg body wt/min (sit-ups, bicycling, swimming, tennis) may result in physiological effects more beneficial than lower level activities even when total caloric expenditure is the same. Age in general results in sharp drop-off in the high energy activities which require socialization (having a partner) and retention of only those activities which can be performed solo (sit-ups). The maintenance of physical activity in the aged would thus be promoted by emphasis on social interaction opportunities. Total activity calories were highly correlated with multiple measures of lean body mass (r values of 0.27 to 0.41, independent of age).

The pathophysiology of airway obstruction comprises a wide variety of anatomic and physiologic alterations. Because of the limitations of current methods of study, most of the physiological alterations are poorly or only partially understood. Measuring the distribution of transit times during forced expiration provides a measure of the degree of uniformity of expiratory flow, and therefore is a valuable tool in understanding the pattern of airflow obstruction in diseases which have limitation of flow as their main component. In allergic bronchospasm it has been proposed that the release of histamine results in afferent vagal stimulation leading to reflect bronchoconstriction, and that asthmatic individuals have a "hypersensitive" response to these stimuli. Therefore pulmonary function was assessed using conventional methods (single breath N_2 washout and forced expiratory spirograms) before and after bronchial challenge with ragweed antigen and methacholine HCl, a parasympathomimetic agent capable of producing changes in bronchomotor tone. The study demonstrated not only a difference in the degree of the bronchospastic response in the asthmatic group, but also a difference in the pattern of airway obstruction. In 55 nonsmoking males aged 20 to 70, although mean transit times did not change with age, the degree of dispersion or non-uniformity of washout increased with age significantly.

The renal response to moderate water deprivation was investigated in ninety-eight participants in the Baltimore Longitudinal Study of Aging. All subjects were free of recent or remote renal disease of any type, diabetes mellitus, coronary or cerebro-vascular disease, urinary tract infection or the administration of medications known to affect renal function. Measurements of urine flow and osmolality were obtained at 3, 6, 9 and 12 hours after the onset of water deprivation. Plasma osmolality was measured at 12 hours. At the onset of the study, subjects were moderately water deprived (urine osmolality 939 ± 26 [SEM] mOsm/L), with no significant age change in urine flow or osmolality. Data from the last six hours of water deprivation showed a significant positive correlation ($R = 0.55$, $p<0.001$) between age and urine flow, and a significant inverse age relationship with urine osmolality ($R= -0.47$, $p<0.001$). Since the degree of impairment with age in the ability to decrease urine flow was greater than the relative inability to concentrate urine, osmolar clearance actually increased with age ($R= 0.48$, $p<0.001$). This age-related impairment in fluid and electrolyte homeostasis may explain the propensity for volume depletion in elderly subjects.

Since the inception of the Baltimore Longitudinal Study, the drug, antipyrine, has been administered intravenously to estimate total body water (TBW). The data required for estimate of TBW can also be utilized to estimate plasma half-life ($T_{\frac{1}{2}}$) of the drug. In 516 subjects, there is a highly significant decrease in $T_{\frac{1}{2}}$ with age. The younger subjects (20-39 yr) show a half-life 39% greater than the older subjects (60-79 yr). Data on drug kinetics and age are fragmentary and therefore these age differences in $T_{\frac{1}{2}}$ of antipyrine, a marker drug for microsomal enzyme degradation, are of great interest.

Studies of dermatoglyphics and other genetic markers have continued along several lines. In the area of clinical medicine, statistical analyses revealed significant relationships between the disease entities of leukemia, Down's Syndrome and deletion of the long arm of the 18 chromosome, and certain dermatoglyphic characteristics. Of special interest were the investigations of palmar creases which have been reclassified. Leukemia and Down's Syndrome were both shown to be associated with simian and Sydney lines. The "thenar" line was also shown to be genetically determined.

Dermatoglyphic characteristics have been found to be governed for the most part by multiple genes and complex types of inheritance. Consequently, they are not readily vulnerable to the influence of selection or genetic drift due to isolation or migration. Thus, the incidence of dermatoglyphic characteristics found in different populations may reveal the nature of the relationships existing between populations in the remote as well as the more recent past.

In Cyprus, similarities found with respect to dermatoglyphics and to thalassemia between peoples in the highlands and the lowlands are indicative of an ancient relationship, while wide differences in blood groups suggest recent changes resulting from isolation and genetic drift among highland peoples, and from migration and admixture among the lowland people. Extensive reviews were completed for the Austral-asian populations, Caucasians, Negroes and the Amerindians. An atlas of the worldwide distribution of the dermatoglyphic features has also been completed and is in press.

In New Guinea, the Fore (a people affected with Kuru) had different dermatoglyphics and also blood groups than their neighbors, the Anga. The findings indicate ancient and persistent genetic differences due to mechanisms affecting isolation. Among the Quechuas of Peru, dermatoglyphic differences were evident while serological factors were quite alike. In contrast to the Cyprus situation, this latter finding is suggestive of ancient genetic differences which have largely disappeared because of more recent admixture.

Twin studies have demonstrated for the first time that mother-fetus incompatibility, due to the Xg^a blood group may result in fetal loss. Because of the sex-linked inheritance of the Xg^a, the fetal loss is confined only to the female fetus. The twin data confirmed findings from the leukemia studies that the simian and Sydney palmar creases are familial and the "thenar" line is inherited.

In an effort to improve the efficiency of routine urinalysis as a screening technique for the presence of renal diseases, we have studied the effects of urinary pH, osmolality and centrifugation time on the number of casts seen in the urinary sediment. Samples of urine from 6-10 patients with a variety of renal diseases were examined. Counts were performed using a Fuchs-Rosenthal chamber. Osmolality and pH were varied within the normal range by the addition of HCl, NaOH, or NA H_2PO_4 and urea or water respectively. There was a highly significant inverse relationship between urinary pH and the number of casts seen. Cast counts dropped progressively to only 57% of the original number as urinary pH rose from 4.5 to 7.5. Centrifugation time (1-30 min) and osmolality (97-821 mosm/l) did not influence the cast counts. Thus, in vitro acidification of urine specimens which cannot be examined immediately prevents degradation of casts and increases the likelihood of their identification during routine urinalysis.

In collaborative studies with scientists at the Johns Hopkins University, two studies on amino acid metabolism in man were carried out. In the first study, the rate of enteric factors in mediating the insulin response to oral amino acids (AA) was studied. AA tolerance tests were performed in obese patients before and 4 months after small bowel bypass. A mixture containing leucine, glycine, and lysine were given. The peak increase in immunoreactive insulin (IRI) after AA ingestion was 81 μU/ml preoperatively but only 18 μU/ml postoperatively. However, the peak increment in AA preoperatively was greater than the postoperative peak increment. To prove that the lower postoperative IRI was not due to the lower plasma AA levels after AA ingestion, preoperative subjects were given 40% of the standard AA load. Despite a peak increment in AA equivalent to that achieved postoperatively, the peak Δ IRI, 75 μU/ml was significantly greater ($p<.005$). To show that the pancreatic β cell could respond to AA stimulation postoperatively the standard AA load was given intravenously. The peak Δ IRI was 170 μU/ml preoperatively. These values are not significantly different. Thus, despite a marked reduction in the β cell response to oral AA after bypass, IRI release following intravenous AA administration is not impaired. These studies suggest that bypassing the small intestine diminishes the release of an enteric factor(s) which augments the insulin response to oral AA.

In the second collaborative study, the effect of a 60 hour fast and of glucagon on peripheral tissue metabolism was studied. Metábolic effects of glucagon or extrahepatic tissues in man have been postulated based generally on studies employing supraphysiologic glucagon concentrations. In this investigation glucagon was infused into the brachial artery to achieve a physiologic increment in hormone concentration within the forearm and effects on muscle and adipose tissue metabolism were quantitated. Seven subjects were studied postabsorptively and seven after a 60 hour fast when the plasma insulin/glucagon ratio was low and increased forearm tissue glucagon sensitivity was anticipated. In postabsorptive subjects a 2 hour intra-arterial glucagon infusion (400-600 pg/kg/min) elevated local concentrations by 1156 pg/ml. Glucagon did not alter muscle balance of glucose or amino acids (acidics and neutrals), or adipose tissue glycerol balance. Fasting reduced the basal insulin (13.0 to 7.5 μU/ml) and increased glucagon (116 to 135 pg/ml) significantly. Muscle output of eight amino acids increased significantly over postabsorptive values, averaging 87% for threonine, glycine, alanine, methionine, isoleucine, and tyrosine. α-Aminobutyrate release increased twelve fold. Serine, taken up postabsorptively, was released in large amounts. In these fasted subjects the 2 hour glucagon infusion elevated glucagon locally by 748 pg/ml but was without effect on muscle balance of glucose, lactate, amino acids, or acetoacetate. Glucose, lactate, FFA, and glycerol balances across adipose tissue were also unchanged. Local and systemic insulin concentrations were unchanged during all glucagon infusions. Thus, physiologic increments in glucagon are without effect on muscle and adipose tissue metabolism in postabsorptive or 60 hour fasted man. Short term starvation is characterized by a dramatic increase in muscle amino acid release in contrast to the conservation of muscle nitrogen accompanying prolonged starvation.

Investigations have begun on a remarkable kindred with familial hemochromatosis, including approximately 80 members in four generations. This is the largest kindred studied and represents an unusual opportunity to investigate several unresolved questions in this field: Specific emphasis is being placed on determining (1) the natural history of the pre-cirrhotic or latent phase of hemochromatosis, (2) the utility of the serum iron as a screening test for excess iron stores, (3) the influence of alcohol intake on the severity of the hepatic injury from iron excess and (4) the etiology of the abnormal carbohydrate metabolism associated with hemochromatosis. A number of studies are performed: oral glucose tolerance, serum iron, liver function tests, twenty-four hour urinary iron excretion after the administration of a chelating agent, and percutaneous liver biopsy. Preliminary results show a high prevalence of excess iron stores (40%), with a pattern of inheritance consistent with an autosomal dominant trait. Serum iron has been found to be an unreliable index of iron stores, and several young adults with repeatedly normal serum irons have been found to have definitely increased hepatic iron. Twenty-four hour urinary iron after desferroximine excretion has not correlated with serum or hepatic iron, and is not useful as a diagnostic test. Thus, liver biopsy seems justified in family members in order to diagnose the disease in an early phase, since venesection therapy can be instituted prior to significant structural damage from iron storage.

One of the participants in our Longitudinal Study of Aging was diagnosed as a case of Fabry's Disease (glycosphingolipidosis), an x-linked disorder characterized by ceramide trihexosidase deficiency. He and two other cases have been studied and reported together since they all showed abnormal atrioventricular conduction. Electrogram of the His bundle showed an abnormally prolonged potential. This may well be due to the effects of deposition of glycolipid in the conducting tissue, a unique finding.

Differences between young and old subjects have been described for a number of hormones. There is however very little information on the mechanism underlying these differences. An extensive study of adenylate cyclase has therefore been undertaken in the rat. The studies have proven to be very complex and have opened some interesting areas of cyclase metabolism. First, the hepatic epinephrine-responsive cyclase is increased in senescence but glucagon cyclase does not change. There is some evidence that the mechanism of this difference may reside in age-related alteration of the characteristics of the cell membrane in senescence. A marked difference in the quantitative and qualitative characteristics of cyclase from homogenates and particulates has been studied. This observation has important implications for the tissue assay of adenylate cyclase generally and for aging specifically. A role for phospholipid in this change is being investigated. The age-related change of adipose tissue cyclase is being studied in isolated fat cell ghosts. To date the results suggest an age-related loss of functional cell membrane mass with retention of cyclase in the remaining membrane. Adenylate cyclase of rat liver and fat cells has been shown by us to be activated not only by F^-, as previously believed, but by other anions as well ($F>Cl>Br>I$). This finding clearly relates cyclase to other anion-activated enzymes. Since a number of such enzymes have been recently studied in pure form, these enzymes may serve as models for cyclase and provide insight into cyclase action.

The chemistry of renin has been further characterized in the past year. It has been demonstrated for the first time that renin is chemically similar to the "acid" proteases in having an aspartic acid active center (inhibition by diazo-Cu^{++}). Also the kinetics of renin inhibition by pepstatin has been defined. Classical non-competitive inhibition by this most potent inhibitor (10^{-10} M) is similar to our earlier demonstration of non-competitive inhibition by other peptides. Pepstatin was used to estimate renin purity by kinetic means and to calculate renin concentration in plasma.

Serial No. HD-CP 7
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Metabolism Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The kinetics of glucose and insulin metabolism in man

Previous Serial Number: HD-CP 7(c)

Principal Investigators:	Jordan D. Tobin Reubin Andres J. Harold Helderman Elizabeth A. McGuire
Other Investigators:	*Mones Berman
Cooperating Units:	*National Cancer Institute Baltimore City Hospitals

Man Years:

Total:	3.7
Professional:	2.2
Other:	1.5

Project Description:

Objectives: These studies are aimed at delineating various parameters of insulin release, distribution, and metabolism in man and the changes in such parameters that occur in altered physiologic states, including aging, diabetes mellitus, and obesity. Because of insulin's role as a prime determinant of glucose utilization, a definition of insulin kinetics will provide a more precise understanding of how the metabolism of glucose is controlled. Current studies are being performed to develop an insulin kinetic model, a glucose kinetic model, and to describe the homeostatic relationship between those two models in normal, diabetic, and obese man of differing ages. From such an analysis, important physiologic controls will be described and clues to pathogenesis of altered metabolic states may be forthcoming.

Methods Employed: The glucose clamp technique was utilized to maintain human subjects at basal arterial blood glucose concentrations after experimental perturbations of the arterial plasma insulin level. The clamp technique employs a controlled variable-rate IV infusion of glucose solution with rapid measurement of blood glucose concentration by an automated method. A negative feed-back formula is applied at 5 minute intervals in order to servo-correct the glucose infusion rate; thus, the blood glucose concentration is maintained at euglycemic levels. The amount of infused glucose, an index of glucose utilization, provides an estimate of sensitivity to exogenous insulin.

Intravenous infusions of insulin were given by 2 techniques: (1) single shot intravenous injection of .025 crystalline porcine insulin per kg body weight or (2) prime plus continuous infusion at 1 or 2 mU per kg body weight per minute. The prime was given over a 10 minute period such that the total insulin infused in that time was twice the rate infused continuously from 10 to 80 minutes. The infusion during the 10 minute prime was at a logarithmically falling rate which approached the continuous infusion rate asymptotically. The goal of this technique was to create a nearly instantaneous square wave of insulin concentration at a new steady state level.

In some studies glucose-1-^{14}C was injected as a primed continous infusion during the pre-clamp period to achieve a steady state specific activity and specific activity of glucose (corrected for metabolites and recycled glucose by established procedures) was followed after the administration of insulin. Such radioactive glucose data allows determination of endogenous glucose production and of glucose utilization as a means of analyzing insulin action.

Plasma immunoreactive insulin concentrations, plasma glucose radioactivity, and plasma glucose concentrations were utilized in mathematical modeling of insulin kinetics and glucose kinetics by the SAAM program of Berman and Weiss to determine compartmental dimensions, transfer rates, and to investigate the mechanisms by which insulin regulates glucose utilization.

Major Findings: Two papers were published on the glucose and the insulin subsystems. Modelling this year has been directed at techniques for use of arterialized venous blood samples (from heated hand), thus avoiding arterial punctures, and at an analysis of the effect of ordinary antecubital venous blood samples on estimates of kinetic parameters. Almost all previous work using labelled glucose in man has not considered this artefact of blood sampling. Triply-cannulated subjects (brachial artery, arterialized venous, and antecubital venous) provided data which were analyzed by the SAAM 25 program. The two types of venous systems can be converted to an arterial system via 2-compartment models. The arterialized venous system has 92% of its flow going through an extremely rapid compartment as compared to only 73% of the flow in the antecubital vein. Arterialized venous blood thus can be readily converted mathematically to arterial blood with little error; without arterialization, i.e., with usual antecubital venous blood sampling, artefacts in kinetic modeling are very large.

Significance to Bio-Medical Research and the Program of the Institute: The remarkable prevalance (50%) of abnormal glucose tolerance tests in the older population of the United States coupled with the increased morbidity and mortality of patients with diabetes mellitus demands a delineation of the effects of aging on the pathophysiology of carbohydrate metabolism. These studies in man define kinetic parameters in the glucose-insulin homeostatic system and will apply such analysis to patients with disorders in that system.

Proposed Course of Project: Altered physiological and pathological states (age, obesity, diabetes, fasting, etc.) will be studied.

Honors and Awards: None

Publications:

Sherwin, R. S., Kramer, K. J., Tobin, J. D., Insel, P. A., Liljenquist, J. E., Berman, M., and Andres, R.: A model of the kinetics of insulin in man. J. Clin. Invest. 53: (May), 1974.

Insel, P. A., Kramer, K. J., Sherwin, R. S., Liljenquist, J. E., Tobin, J. D., Andres, R., and Berman, M.: Modeling the insulin-glucose system in man. Fed. Proc. 33: (June), 1974.

Serial No. HD-CP 20
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Metabolism Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Uremia and Carbohydrate Intolerance

Previous Serial Number: Same

Principal Investigator: Ralph A. DeFronzo

Other Investigators: Reubin Andres
Jordan D. Tobin
John W. Rowe
Dan Sapir*

Cooperating Units: Johns Hopkins Hospital
*Renal Division

Man Years:

Total: 0.2
Professional: 0.2
Other: 0.0

Project Description:

Objectives: Glucose intolerance occurs in greater than 50% of uremic individuals. However, despite numerous investigations the underlying mechanism (s) has not been agreed upon. Before the advent of dialysis and transplantation the finding of abnormal glucose tolerance in patients with chronic renal disease had little bearing on patient management. However, the availability of these newer therapeutic measures has created new significance for uremic carbohydrate intolerance. Where diabetics have been accepted for chronic dialysis or transplantation, the incidence of serious complications and mortality has been 4-5 times higher than in nondiabetics. Thus, it has become important to distinguish between true diabetes mellitus and uremia-induced glucose intolerance.

Because of the diagnostic and therapeutic implications of carbohydrate intolerance to the patient with chronic renal disease, it was felt important

to summarize the large body of literature that has accumulated on the subject of uremia-induced carbohydrate intolerance and to attempt to explain apparent discrepancies reported by different investigators. This was done and our review paper has been published in Medicine.
In this paper, we propose the hypothesis that glucose intolerance occurs in those individuals who are unable to augment insulin secretion sufficiently to overcome the peripheral antagonism to insulin that exists in uremic individuals. The studies to be reported here are designed to test this hypothesis.

Methods Employed: Carbohydrate tolerance was evaluated in eight chronically uremic subjects accepted into our hemodialysis program employing the servo-control "glucose clamp" technique previously described by us and with standard intravenous and oral glucose tolerance tests.

Major Findings: All eight subjects have had pre- and post-dialysis hyperglycemic and euglycemic clamps. An interesting additional conclusion (since last year's report) emerged with the completion of the study: there is a very strong negative correlation ($r = 0.931$, $p < 0.001$) between the change in tissue sensitivity to insulin post-dialysis and the change in beta cell sensitivity to glucose. This correlation is consistent with other conditions which result in tissue resistance to insulin such as obesity, adrenal steroids, pregnancy, etc. These conditions, which lead to tissue insensitivity, also result in increased responsiveness of the beta cell to glucose. Thus, when uremics are dialyzed, they show a recovery of their tissue sensitivity and a strongly negative correlation of this change with beta cell sensitivity. Three of the eight subjects however (the three with the least change in tissue sensitivity) showed an actual increase in beta cell sensitivity to glucose. These data support a hypothesis which we proposed in our recent review of the uremia problem, to wit, that there are dual metabolic effects of uremia on the beta cell: (1) a direct inhibitory effect and (2) an indirect stimulatory effect secondary to the effect of uremia on tissue sensitivity. This work is being reported at the Plenary Session of the annual meeting of the American Society for Clinical Investigation.

Proposed Course of the Project: Kinetic modeling of the insulin system will be undertaken from data on the euglycemic (insulin-infusion) clamp studies.

Honors and Awards: None

Publications: None

Serial No. HD-CP 12
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Metabolism Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Cytoxan-induced water intolerance

Previous Serial Number: Same

Principal Investigator: Ralph A. DeFronzo

Other Investigators:
Paul J. Davis**
Michael Colvin*
Hayden Braine*
Gary Robertson***

Cooperating Units:
Baltimore City Hospitals
* Oncology Service
** Endocrine Service
***Indianapolis VA Hospital, Indianapolis, Indiana

Man Years:

Total:	0.0
Professional:	0.0
Other:	0.0

Project Description: Project has been completed and results have been published.

Publications:

DeFronzo, R. A., Abeloff, M., Braine, H., Humphrey, R. L., and Davis, P. J.: Renal dysfunction after treatment with isophosphamide. Cancer Chemotherapy Reports. 58:No. 2 (May-June), 1974.

DeFronzo, R. A., Colvin, O. M., Braine, H., Robertson, G. L., and Davis, P. J.: Cyclophosphamide and the kidney. Cancer. 33: 483-491, 1974.

Serial No. HD-CP-27
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Metabolism Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Effect of age on metabolism

Previous Serial Number: HD-AG 10 (c)

Principal Investigators: Reubin Andres
Jordan D. Tobin
John W. Rowe

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	5.0
Professional:	1.9
Other:	3.1

Project Description:

Objectives: This project is part of the long-term prospective study of human aging, the Longitudinal Study of the Gerontology Research Center. The complex interplay of aging, carbohydrate metabolism and diabetes mellitus offers a prototype of one of the serious challenges to clinical gerontology, the differentiation of normal aging changes from specific disease states. The objectives of this project are: (1) to describe cross-sectional changes during the entire adult span of life in performance on the commonly-used clinical tests for the diagnosis of diabetes, (2) to provide age-adjusted standards of normality for these tests, (3) to define the relative sensitivity and specificity of these tests, (4) to establish the role of certain experimental variables which influence performance on these tests, (5) to investigate the mechanisms underlying the age changes, and (6) to evaluate the significance of the decline in tolerance [a] by noting the influence of poor performance on mortality and [b] by testing for correlations between performance and certain other variables known to be clearly associated with the overt diabetic state.

Methods Employed: Subjects are primarily those in the Longitudinal Study supplemented by college students and laboratory and hospital employees to increase the sample size of the 3rd and 4th decades. Subjects are given one of the following tests per visit: oral glucose tolerance (OGTT), intravenous glucose tolerance test (IVGTT), cortisone glucose tolerance (CGTT), or intravenous tolbutamide response (TRT). On the fifth visit the cycle is re-started. Diabetic patients are available primarily from the Baltimore City Hospitals Out-Patient Department; "chemical" diabetes and a small number of overt diabetics are available from the Longitudinal Study Group.

Major Findings: The effect of insulin or urinary electrolyte excretion in the absence of a change in filtered glucose was examined in 6 healthy volunteers undergoing water diuresis. After a 120 min control period, plasma insulin concentration was abruptly increased and maintained between 98-193 uU/ml for 120 min by continuous IV insulin infusion (2mU/kg body wt/min). Plasma glucose concentration was maintained within 96-99% of the fasting level by varying the rate of concomitant IV glucose administration. During insulin administration, P_{Na} and P_{Osm} were unchanged and P_K decreased ($p<.001$). $U_{Na}V$ decreased from 401 to 213 uEq/min ($p<.02$); U_KV decreased from 66 to 21 uEq/min ($p<.005$); and $U_{Ca}V$ increased from 128 to 183 ug/min ($p<.02$). C_{IN}, C_{PAH}, and urine flow were unchanged; C_{Osm} decreased from 4.9 to 3.3 ml/min ($p<02$) and was associated with an increase in C_{H20} from 10.6 to 13.0 ml/min ($p<.01$). Plasma aldosterone was unchanged and plasma glucagon concentration decreased slightly from 97 to 74 pg/ml. Control subjects restudied under similar conditions but without insulin administration showed no changes in urinary electrolyte excretion, C_{Osm}, or C_{H20}. These studies demonstrate an effect of insulin on $U_{Na}V$ which can not be attributed to changes in the filtered load of solute, GFR, RPF, or to changes in plasma aldosterone, glucagon, or glucose concentrations. The data are consistent with a direct effect of insulin on tubular sodium reabsorption and suggest that this enhanced sodium reabsorption occurs in the diluting segment of the renal tubule.

Hypoglycemia is known to induce the secretion of multiple hormones, including glucagon (GLN), growth hormone (HGH), cortisol (COR), and catecholamines (CAT). Whether the stimulus to these responses is the absolute glucose concentration or the rate of change in glucose concentration (or both) is not clear. Experimental design: Brachial arterial blood was monitored in 5 normal male volunteers aged 24-37 years (1) during the rapid induction and maintenance of hyperglycemia at 200 mg/100 ml above basal for 1 hour using a servo-controlled intravenous glucose infusion (glucose clamp technique) and (2) during a rapidly falling glucose phase after the glucose infusion was discontinued. Results: Blood glucose (BG) fall averaged 146 mg/100 ml by 20 minutes (110-170). Despite this precipitous drop, no increase in level of any of the four counterregulatory hormones occurred. Basal BG levels were reached by 26-47 minutes; the BG nadir ranged from 45 to 64 mg/100 ml. Hormonal responses as BG reached hypoglycemic levels occurred in 4/5 subjects for HGH and 3/3 for CAT, but only 1/5 for COR. GLN levels were suppressed during the hyperglycemic plateau in 4/5; 2 of these showed recovery to basal levels only and 2 exceeded basal levels during hypoglycemia. Conclusion: The stimulus to the release of counterregulatory hormones was hypoglycemia per se; none of the four hormones responded to a rapidly falling glucose concentration.

In collaborative studies with scientists at the Johns Hopkins University, two studies on amino acid metabolism in man were carried out. In the first study, the rate of enteric factors in mediating the insulin response to oral amino acids (AA) was studied. AA tolerance tests were performed in obese patients before and 4 months after small bowel bypass. Following surgery 15 inches of jejunum and 5 of terminal ileum were in continuity. A mixture containing 75 mmoles each of leucine, glycine, and lysine were given. The peak increase in immunoreactive insulin (IRI) after AA ingestion was 81 ± 16 μU/ml preoperatively (n=13) but only 18 ± 4 μU/ml postoperatively (n=10). However, the peak increment in AA preoperatively was greater than the postoperative peak increment (Δleu=775 ± 53 vs. 443 ± 48 μM/L). To prove that the lower postoperative IRI was not due to the lower plasma AA levels after AA ingestion, 6 additional preoperative subjects were given 40% of the standard AA load. Despite a peak increment in AA (Δleu= 397 ± 69 μM/L) equivalent to that achieved postoperatively, the peak Δ IRI (75 ± 16 μU/ml) was significantly greater ($p<.005$). To show that the pancreatic β cell could respond to AA stimulation postoperatively the standard AA load was given intravenously. The peak Δ IRI was 170 ± 26 μU/ml preoperatively (n=6). These values are not significantly different. Thus, despite a marked reduction in the β cell response to oral AA after bypass, IRI release following intravenous AA administration is not impaired. These studies suggest that bypassing the small intestine diminishes the release of an enteric factor(s) which augments the insulin response to oral AA.

In the second collaborative study, the effect of a 60 hour fast and of glucagon on peripheral tissue metabolism was studied. Metabolic effects of glucagon on extrahepatic tissues in man have been postulated based generally on studies employing supraphysiologic glucagon concentrations. In this investigation glucagon was infused into the brachial artery to achieve a physiologic increment in hormone concentration within the forearm and effects on muscle and adipose tissue metabolism sought. Seven subjects were studied postabsorptively, and seven after a 60 hour fast when the plasma insulin-glucagon ratio was low and increased forearm tissue glucagon sensitivity anticipated. In postabsorptive subjects a 2 hour intra-arterial glucagon infusion (4-0-600 pg/kg/min) elevated local concentrations by 1156 pg/ml. Glucagon did not alter muscle balance of glucose or amino acids (acidics and neutrals), or adipose tissue glycerol balance. Fasting reduced basal insulin (13.0 to 7.5 μU/ml) and increased glucagon (116 to 135 pg/ml) significantly. Muscle output of eight amino acids increased significantly over postabsorptive values, averaging 87% for threonine, glycine, alanine, methionine, isoleucine, and tyrosine. α-Aminobutyrate release increased twelve fold. Serine, taken up postabsorptively, was released in large amounts. In these fasted subjects the 2 hour glucagon infusion elevated glucagon locally by 748 pg/ml but was without effect muscle balance of glucose, lactate, amino acids or acetoacetate. Glucose, lactate, FFA, and glycerol balance across adipose tissue were also unchanged. Local and systemic insulin concentrations were unchanged during all glucagon infusions. Thus, physiologic increments in glucagon are without effect on muscle and adipose tissue metabolism in postabsorptive or 60 hour fasted man. Short term starvation is characterized by a dramatic increase in muscle amino acid release in contrast to the conservation of muscle nitrogen accompanying prolonged starvation.

The renal response to moderate water deprivation was investigated in ninety-eight participants in the Baltimore Longitudinal Study of Aging. All subjects were free of recent or remote renal disease of any type, diabetes mellitus, coronary or cerebro-vascular disease, urinary tract infection or the administration of medications known to affect renal function. Measurements of urine flow and osmolality were obtained at 3, 6, 9 and 12 hours after the onset of water deprivation. Plasma osmolality was measured at 12 hours. At the onset of the study, subjects were moderately water deprived (urine osmolality 939 ± 26 [SEM] mOsm/L), with no significant age change in urine flow or osmolality. Data from the last six hours of water deprivation showed a significant positive correlation ($R = 0.55$, $p<0.001$) between age and urine flow, and a significant inverse age relationship with urine osmolality ($R = -0.47$, $p<0.001$). Since the degree of impairment with age in the ability to decrease urine flow was greater than the relative inability to concentrate urine, osmolar clearance actually increased with age ($R = 0.48$, $p<0.001$). This age-related impairment in fluid and electrolyte homeostasis may explain the propensity for volume depletion in elderly subjects.

Since the inception of the Baltimore Longitudinal Study, the drug, antipyrine, has been administered intravenously to estimate total body water (TBW). The data required for estimate of TBW can also be utilized to estimate plasma half-life ($T_{\frac{1}{2}}$) of the drug. In 516 subjects, there is a highly significant decrease in $T_{\frac{1}{2}}$ with age. The younger subjects (20-39 yr) show a half-life 39% greater than the older subjects (60-79 yr). Data on drug kinetics and age are fragmentary and therefore these age differences in $T_{\frac{1}{2}}$ of antipyrine, a marker drug for microsomal enzyme degradation, are of great interest.

In an effort to improve the efficiency of routine urinalysis as a screening technique for the presence of renal disease, we have studied the effects of urinary pH, osmolality and centrifugation time on the number of casts seen in the urinary sediment. Samples of urine from 6-10 patients with a variety of renal diseases were examined. Counts were performed using a Fuchs-Rosenthal chamber. Osmolality and pH were varied within the normal range by the addition of HCl, NaOH, or $Na\ H_2PO_4$ and urea or water respectively. There was a highly significant inverse relationship between urinary pH and the number of casts seen. Cast counts dropped progressively to only 57% of the original number as urinary pH rose from 4.5 to 7.5. Centrifugation time (1-30 min) and osmolality (97-821 mosm/l) did not influence the cast counts. Thus, in vitro acidification of urine specimens which cannot be examined immediately prevents degradation of casts and increases the likelihood of their identification during routine urinalysis.

Investigations have begun on a remarkable kindred with familial hemochromatosis, including approximately 80 members in four generations, scattered throughout the middle Atlantic states. This is the largest kindred with familial hemochromatosis to be studied and represents an unusual opportunity to investigate several unresolved questions in this field. A number of studies are performed: oral glucose tolerance, serum iron, liver function tests, twenty-four hour urinary iron excretion after the administration of a chelating agent, and percutaneous liver biopsy. Light and electron microscopic studies are performed on the biopsy specimens.

Specific emphasis is being placed on determining (1) the natural history of the pre-cirrhotic or latent phase of hemochromatosis, (2) the utility of the serum iron as a screening test for excess iron stores, (3) the influence of alcohol intake on the severity of the hepatic injury from iron excess and (4) the etiology of the abnormal carbohydrate metabolism associated with hemochromatosis.

Preliminary results of investigations on a portion of the kindred show a high prevalence of excess iron stores (40%), with a pattern of inheritance consistent with an autosomal dominant trait. Serum iron has been found to be an unreliable index of iron stores, and several young adults with repeatedly normal serum irons have been found to have definitely increased hepatic iron. Twenty-four hour urinary iron after desferroximine excretion has not correlated with serum or hepatic iron, and is not useful as a diagnostic test. Thus, liver biopsy seems justified in family members in order to diagnose the disease in an early phase, since venesection therapy can be instituted prior to significant structural damage from iron storage.

One of the participants in our Longitudinal Study of Aging was diagnosed as a case of Fabry's Disease (glycosphingolipidosis), an x-linked disorder characterized by ceramide trihexosidase deficiency. He and two other cases have been studied and reported together since they all showed abnormal atrioventricular conduction. Electrogram of the His bundle showed an abnormally prolonged potential. This may well be due to the effects of deposition of glycolipid in the conducting tissue, a unique finding.

Proposed Course of Project: (1) Analyze the routine diagnostic tests for diabetes through the end of Cycle G (June 30, 1973). These computations in association with the other variables measured in the Longitudinal Study will provide the evidence to meet goals (1) to (4) and goal (6) as outlined in the Objectives section. The interrelated variables are the following: (a) glucose and insulin responses on the clinical tests, (b) serum lipids, (c) body composition, especially lean body mass and obesity, (d) physical activity, (e) dietary factors, and (f) presence of atherosclerotic disease.

Honors and Awards: Reubin Andres

1. Superior Service Award, Dept. H.E.W.
2. Elected as Chairman, Clinical Medicine Section, Gerontological Society.

3. Invited lecturer:

   a. Brooklyn Society of Internal Medicine, Downstate Medical College, May 10, 1973.

   b. Fifth Annual Biology of Aging Short Course, University of Minnesota, Duluth, Minnesota, August 13-17, 1973.

   c. The Medical College of Pennsylvania, November 29, 1973.

   d. Baltimore City Hospitals Bicentennial Program co-sponsored by American Geriatrics Society, December 1, 1973.

   e. College of Physicians and Surgeons, Columbia University, December 5, 1973.

   f. Seminar on Theoretical Aspects of Aging, University of Miami, February 8, 1974.

Honors and Awards: Jordan D. Tobin
Invited lecturer:

   a. Symposium on Diseases of the Aged at Washington County Hospital, Hagerstown, Maryland, sponsored by the American Geriatrics Society, November 1, 1973.

   b. Third Congress on the Quality of Life--the Later Years sponsored by the American Medical Association, Chicago, Illinois, April 1-3, 1974.

   c. Wayne State University, Institute of Gerontology, April 19, 1974.

Publications: None

Serial No. HD-CP 30
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Metabolism Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The effect of age on the pharmacokinetics and pharmacologic effects of ethanol in man

Previous Serial Number: New Project

Principal Investigators: Robert E. Vestal
Betty Ann Robertson*

Other Investigators: J. Harold Helderman
[redacted]
Jordan D. Tobin
Reubin Andres
David L. Arenberg*
Esteban Mezey**
Gary L. Robertson***
Ronald Krauss****

Cooperating Units:
* Laboratory of Behavioral Science, GRC, NICHD
** Alcohol Research Unit, Baltimore City Hospitals
*** Indianapolis VA Hospital, Indianapolis, Indiana
**** Molecular Disease Branch, NHLI

Man Years:

Total:	1.5
Professional:	1.1
Other:	0.4

Project Description:

Objectives: The goal of these studies is to examine under conditions of acute ethanol administration the effect of age on the following: (1) the kinetics of distribution and elimination of ethanol, (2) the effect on insulin, carbohydrate and lipid metabolism, (3) the effect on posterior pituitary function and free water clearance, (4) the effect on psychomotor and cognitive performance.

Methods Employed: Ethanol is administered intravenously as a 15% solution in a dose of 400 mg/m^2/min over 60 minutes (approximately 0.6 gm/kg) following an overnight fast with fluid restriction. Blood samples are obtained for determination of ethanol, glucose, insulin, sodium, osmolality, arginine vasopressin over a 5 hour period. Voided urine samples are collected for determination of ethanol and free water clearance. Four hours following infusion sodium heparin (10u/kg) is rapidly infused and appropriate samples are obtained for cholesterol, triglycerides and post-heparin lipolytic activity. Serial tests of psychomotor and cognitive performance are administered before, during and following ethanol administration.

Major Findings: Results are not yet available for analysis. Infusions have been completed in 6 subjects, age 20-49 and 14 subjects, age 65-92. Additional subjects in young, middle and old age groups are required to complete an adequate sample for cross-sectional age comparisons.

Significance to Bio-Medical Research: The importance of age in the clinical pharmacology of drugs has been recognized but little studied. Because of its extensive social use and abuse, its clinical importance, and its broad biochemical, physiological, and psychological effects, ethanol was chosen as a drug worthy of investigation.

Proposed Course of Project: This study will be completed by July 1, 1975.

Honors and Awards: None

Publications: None

Serial No. HD-CP 17
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Cardiovascular Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Effect of age on the response to inotropic interventions and hypoxia of rat trabeculae carneae

Previous Serial Number: Same

Principal Investigators: Edward G. Lakatta
Gary Gerstenblith

Other Investigators: Myron L. Weisfeldt
Nathan W. Shock

Cooperating Units: Baltimore City Hospitals; Johns Hopkins Hospital, Division of Cardiology; and School of Veterinary Medicine and Department of Medicine, University of Pennsylvania

Man Years:

Total:	1.8
Professional:	1.4
Other:	0.4

Project Description:

Objectives: The original objectives were: (1) to determine the effect of age on inotropic response to catecholamines of isolated myocardium, (2) to examine the effects of paired stimulation, and (3) to examine the effect of age in the response to hypoxia and reoxygenation. Since there was a significant age difference in the inotropic response to catecholamines, to paired stimulation and in recovery from hypoxia, additional experiments were undertaken to investigate the mechanisms responsible for these observed age differences. These included measurement of the inotropic response to a single high concentration of isoproterenol and calcium, hypoxia and reoxygenation after catecholamine depletion with 6-hydroxydopamine, and measurement of tissue catecholamines. The intracellular action potential of young adult and aged myocardium was also measured, using the microelectrode technique.

Methods Employed: The techniques employed in these experiments were described in detail in the 1972-73 project report.

Major Findings: The major findings can be separated into two categories: (1) the inotropic response to increasing concentrations of norepinephrine was diminished with age. The response to a single high concentration of norepinephrine and isoproterenol was also diminished in the aged (25 mo.) myocardium. In contrast, the response to increasing calcium contractions, including the maximal response to calcium, was identical in young and aged myocardium. These results demonstrate that the inotropic response to catecholamines is diminished in the aged myocardium and that this diminished response is not due to tachyphylaxis or to an age difference in tissue catecholamine uptake. Also, since there is no age difference in response to calcium , it would appear that the age differences after catecholamine lie proximal to the contractile proteins. (2) Contraction duration is prolonged in aged myocardium both in control measurements and during recovery from hypoxia. Although endogenous myocardial catecholamines were diminished in aged myocardium, depletion of endogenous catecholamines with 6-hydroxydopamine failed to obliterate the prolonged contraction duration in aged myocardium. Also, at short pairing intervals, fewer aged muscles generated a second mechanical event. Since there was no difference in electrical refractory period, it was concluded that the aged muscle exhibited greater electro-mechanical dissociation. Prolonged contraction duration and greater electromechanical dissociation in aged myocardium are indicative of a prolonged active state and appear to be due to slower renewal of calcium from the contractile protein.

Significance to Biomedical Research and the Program of the Institute: The project provided the initial data regarding a diminished inotropic response to catecholamines and provided a plausible testable mechanism for the age difference, namely, a diminished increase in calcium availability after catecholamines in aged myocardium. This study also confirmed that contraction duration is prolonged in aged myocardium and demonstrates that this phenomenon is due, at least in part, to prolonged active state.

Proposed Course of the Project: Current objectives are (1) to attempt to correlate the prolonged contraction duration with measurements of calcium sequestration of isolated microsomial preparation. (2) To investigate the effect of age on response to rapid stimulation and to digitalis, an agent which acts by increasing calcium availability from extracellular sources.

Honors and Awards: Preliminary results were presented at the Research Seminar of the Division of Cardiology at Johns Hopkins University.

Publications: None

Serial No. HD-CP 31
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Cardiovascular Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Hemodynamics of the left ventricle and mitral valve in aging man

Previous Serial Number: None

Principal Investigators: Gary Gerstenblith
Edward Lakatta

Other Investigators: Myron Weisfeldt
Nathan Shock

Cooperating Units: Baltimore City Hospitals and Johns Hopkins Hospital, Division of Cardiology

Man Years:

Total:	0.60
Professional:	0.40
Other:	0.20

Project Description:

Objectives: This project is designed to assess, in a non-invasive manner, the hemodynamic performance of the left ventricle and the movement of the mitral valve during rest and in response to stress in the Longitudinal Study participants at the Gerontology Research Center. The time periods for isometric contraction, and relaxation, and electromechanical delay will also be determined.

Methods Employed: The mitral valve, posterior left ventricular and septal wall motions will be recorded by echocardiography while the subject is in the supine position at rest and during blood pressure increase induced by hand grip exercise. Simultaneous EKG, carotid pulse, apex cardiogram, and phonocardiogram traces will also be obtained. Posterior left ventricular wall thickness will be read directly from the record. The change in minor axis dimension between the septum and posterior wall in systole and diastole will be utilized to determine left ventricular volume, ejection fraction, and velocity of circumferential fiber shortening. The intervals between the QRS complex and mitral valve closure, between mitral valve closure and the onset of the carotid upstroke, and between the carotid pulse incisura and mitral valve opening will provide indices of electro-mechanical delay, isometric contraction, and isometric relaxation respectively. The mean rate of mitral valve opening, obtained by computing the slope between the e and f points on the mitral valve echogram will be used as an index of mitral valve motion.

Major Findings: The echocardiograph machine first became available on April 25, 1974 and, therefore, there have been no findings as of this writing.

Significance to Biomedical Research and the Program of the Institute: The echocardiogram provides a non-invasive method of evaluating ventricular function in the longitudinal participants. Studies of the isometric performance of isolated trabeculae carneae from this laboratory suggest that isometric contraction duration is prolonged and that the intrinsic cardiac response to stress is diminished in aged myocardium. It will be possible to assess and quantify the extent to which these phenomena are present in intact, aged men. Undoubtedly, multiple avenues for future investigation will become evident as the study progresses.

Honors and Awards: None

Publications: None

Serial No. HD-CP 18
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Endocrinology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1,1973 through June 30, 1974

Project Tiltle: Hormone receptors and aging: Effects of age on hormone mediated alterations of adenylate cyclase, tissue cyclic AMP and factors controlling these metabolic regulators.

Previous Serial Number: HD-CP-18

Principal Investigators: Robert I. Gregerman
Martin I. Kalish
Barry Cooper (E.O.D. July 1, 1973

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:
Total:
Professional:
Other:

Project Description:

Objectives: Adenylate cyclase is a membrane-bound enzyme which catalyses the conversion of adenosine triphosphate (ATP) to adenosine-3' -5' cyclic monophosphate (cyclic AMP). The enzyme is very widely distributed in nature. It is well established that in initiating these effects various hormones activate adenyl cyclase and thus increase the cellular concentration of cyclic AMP. Cyclic AMP in turn initiates a varity of metabolic effects, depending on the tissue involved. This process is known as the "second messenger" concept of hormone action.

Several studies from other laboratories indicate that aging in animals is accompanied by effects on hormone receptors and adenylate cyclase. In rat adipose tissue sensitivity to lipolytic agents (glucagon; epinephrine) decreases with maturation. No information has been available for post-maturation changes (senescence). Very recent studies indicate a complex pattern of hormone sensitive cyclase (norepinephrine; dopamine) loss in the brain of the senescent rat. Practically no other information concerning the cyclase system is available at this time.

Our present study investigates effects of aging, and especially of senescence, on hormone mediated activation of adenylate cyclase and the production of intracellular cyclic AMP. The information developed will be useful in determining whether the already known alteration of tissue sensitivity to certain hormones is the result of an age-related alteration of the initial step in hormone action. The study will also allow an examintion of the effect of senescence on one aspect of membrane function.

Methods Employed: Adenylate cyclase is assayed by measurement of the generation of [^{32}P]cyclic-AMP from [^{32}P]ATP which is isolated by one of two column procedures. Tissue homogenates, particulates, fat cells, and fat cell "ghosts" are prepared by standard methods.

Major Findings: 1. Adenylate cyclase in liver. The preliminary results reported last year are confirmed and extended. Three, 12 and 24 month old male and female rats have been studied. Liver homogenates from senescent animals show two times more epinephrine responsive cyclase activity than 12 month old animals. The dose-response to epinephrine is unaffected by age, i.e,. half-maximal stimulation and full stimulation occur at the same concentrations of epinephrine regardless of age. In contrast to the epinephrine response, that to glucagon is unaffected by the age of the animal. Non-specific activation by fluoride ion is also unaffected by age.

In contrast to these results with liver homogenates, washed particulate preparations show no age differences. Moreover, these preparations are significantly more sensitive to low concentrations of epinephrine (down to 10^{-14} M) and less sensitive to high concentrations. The simple act of centrifuging a homogenate results in slight loss of activity, but this is accentuated when the particulate is washed. To date we have been unsuccessful in identifying the factor or factors necessary for preservation of activity in particulates. The problem is a major one, not only because of the implications for age-effect, but because the homogenate-particulate difference has not been recognized and is key to the proper assay of cyclase in tissues generally.

Homogenates which are stored at 0° C for 18 hours show an age-dependent loss of epinephrine sensitive cyclase. Homogenates from 3 month animals are most stable, from 12 months less so, while those from 24 month old animals show the greatest loss. Fluoride activation decreases in parallel to the epinephrine responsive enzyme, but glucagon activity is well maintained. Proteinase inhibitors (DFP; Trasylol) are inefective in preventing the loss of cyclase during storage at 0° suggesting that age-related increase of proteases is not responsible for these results. Rather the data suggest an age-depent alteration of liver plasma membrane which results somehow in both greater stimulation of cyclase by epinephrine and increased instability of the cell membrane.

2. Adenylate cyclase in fat cells. Preliminary results indicate that epinephrine sensitve adenylate cyclase of fat cells, expressed as enzyme activity per cell, increases sharply with maturation between 4-8 weeks; thereafter, the enzyme activity decreases progressively. The fall from the 2 month peak to 6 months of age is almost one-half. There is a similar fall to 1 year and a smaller fall thereafter. However, the cyclase activity of cell membranes of all ages examined is unaffected by age when activity is expressed per unit of membrane protein. The implication of this finding appears to be the unexpected finding that aging in adipose tissue of the rat results in a progressive loss of the mass of the fat cell membrane. Cyclase appears to be lost in direct proportion to the loss of membrane protein.

3. Anion activation of adenylate cyclase. Fluoride has long been known to stimulate adenylate cyclase. The mechanism of this ion effect has been obscure, and the phenomenon attributed solely to this particular halide ion; others were thought not to be effective. We have now shown that cyclase of liver and fat is stimulated by chloride, bromide and iodide ions. This finding clearly relates adenylate cyclase to a growing number of other enzymes which are halide (and fluoride) activated and which have been recently studied by physico-chemical means. Our observation is important for an understanding of the mechanism of cyclase activation.

Significance to Bio-Medical Research and the Program of the Institute. These studies should provide an important insight into the mechanisms by which age affects the organism's response to hormones. The results already indicate a selective alteration of cyclase responses during senescence. In addition our studies offer an opportunity to study a membrane-bound enzyme and will thus give an insight into age effects on membrane function. The work is directly related to the mission of the Gerontology Research Center and the Institute to provide new information in the area of research on Aging.

Proposed Course of Project: Attempts will be made to elucidate the mechanism of the age-influenced increase of adenylate cyclase activity in liver. An explanation will be sought for the homogenate-particulate difference in terms of membrane lipid loss. Further studies on the apparent loss of adipose cell membrane mass will be made and may include study of other cell membrane markers. A survery of anion effects on adenylate cyclase of various tissues may be helpful in understanding the mechanism of certain long-known halide anion effects.

Honors and Awards: None

Publications: None.

Serial No. HD-CP 28
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Endocrinology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The biochemistry of renin

Previous Serial Number: HD-AG21 (c)

Principal Investigators: Robert I. Gregerman
Mary M. McKown (resigned 12/31/73)
Hardy J. Chou (E.O.D. 4/1/74)

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:
Total:
Professional:
Other:

Project Description:

Objectives: The highly specific proteinase, renin, is secreted by the kidney in response to a variety of physiologic stimuli. The enzyme is produced in specialized cells of the juxtaglomerular apparatus. Once released into the circulation the enzyme cleaves a decapeptide, angiotensin I, from a plasma glycoprotein. This peptide is then converted to the octapeptide angiotensin II by enzymes in the lung. The physiologic role of angiotensin II appears to involve regulation of the production of the adrenal's major salt retaining hormone, aldosterone. In pathologic states (renovascular stenosis) excessive renin production leads to hypertension. Abnormalities of renin secretion are also involved in other forms of hypertension. The quanitation of plasma renin has assumed a role in the differential diagnosis of hypertensive deseases and in the selection of drugs for the treatment of hypertension. Recent studies indicate that aged subjects have impairments of renin secretion and aldosterone production.

Because of these considerations, our laboratory has been interested in the development of new approaches to the measurement of renin and angiotensin, in the chemistry of the enzyme and its peptide products, and in the physiology of the renin angiotensin-aldosterone system in normal and pathologic aging.

Methods Employed: Previous reports have described our developments of double isotope derivative and labeled polymeric substrate assays for renin.

Major Findings: I. Pepstatin inhibition of human renin. The microbial pentapeptide, pepstatin, has been shown to be an extremely potent inhibitor of renin ($K_I = 10^{-10}$ M). Kinetic studies indicate that the inhibition is reversible and of the non-competitive type. In this regard pepstatin is qualitatively similar to the leucine containing peptides we have previously described but is 4 orders of magnitude more potent. The high affinity of pepstatin for the enzyme allows a novel determination by kinetic means of the purity of renin preparations. We have now applied this procedure for the first time. We have also calculated the maximum theoretical specific activity of renin by this method.

II. Diazoacyl inhibition of human renin. The finding that renin is inhibited by pepstatin, along with certain considerations of its sequence specificity, indicate a similarity to the acid proteinases, especially pepsin and cathepsin D. We have now found that, like these latter two enzymes, renin is inhibited by a diazo reagent (diazoacetylglycine ethyl ester). This finding establishes renin as a proteinase with aspartic acid at its active center.

III. Proteinase activity of human renin preparations. Further studies on this finding, first reported last year, indicates that the proteinase activity is inhibited by pepstatin and the diazoacyl reagent. This finding suggests that such activity may indeed be due to renin and raises further questions concerning renin's specificty.

Significance to Bio-Medical Research and the Program of the Institute: The finding that human renin is inhibited by pepstatin opens the way for development of potent inhibitors of the enzyme for in vivo use. The recognition that renin is an "acid" proteinase raises the issue of renin's possible identity with cathepsin D or a similar enzyme. This consideration can be explored with the idea of obtaining pure human renin from non-renal sources. Our laboratory's continued work in this area may lead to better diagnostic methodology in the hypertension area and possible new theraputic approaches. In addition, our work is relevant to understanding basic aspects of the chemistry of the enzyme.

Proposed Course of Project: We are attempting to synthesize a polymeric pepstatin inhibitor of renin. This should greatly facilitate the in vivo use of pepstatin as a renin inhibitor, presently limited by problems of solubility and short duration of action. Other work will involve the synthesis of a polymeric renin substrate in which the C-terminal portion of the peptide is linked to the polymer through the C-terminal hydroxyl group of serine, in simulation of the natural protein substrate. This material may prove a more specific substrate for renin which will not be attacked by pseudorenin.

Honors and Awards: Dr. Gregerman spoke on the "Chemistry of Renin" at the University of Maryland, Department of Biological Chemistry, April 19, 1974.

Publications: Workman R. J., McKown, M. and Gregerman, R. I., Renin: Inhibition by proteins and peptides. Biochemistry, June, 1974.

Serial Number: HD-CP-23
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Longitudinal Studies of Human Physiology, Biochemistry and Psychology

Previous Serial Number: HD-AG3(c)

Principal Investigators: N. W. Shock, Director
Reubin Andres, Clinical Director
Arthur H. Norris, Coordinator

Other Investigators:

D. Arenberg
B. D. Bricker
M. Butler
B. T. Engel
C. E. Martin
C. C. Plato
J. D. Tobin
A. Zuckerman

Cooperation Units: Baltimore City Hospitals

Dr. Bernice H. Cohen
School of Hygiene & Public Health
The Johns Hopkins University

Dr. James Schlesselman & Dr. Samuel W. Greenhouse
Biometry Branch, NICHD-NIH, Bethesda

Man Years:

Total:	11.50
Professional:	4.55
Other:	6.95

Project Description:

Objectives: This project is designed to: (1) secure replicate measures of physiological, pathological, biochemical and psychological variables on longitudinal study participants at specified intervals; (2) summarize and compare the results of testing in relation to age according to cross-sectional and longitudinal formats; (3) identify characteristics of individual participants which may be related to changes of function over time and to age at death; and, (4) determine whether the data obtained support one or another theory of the mechanisms responsible for age-related functional decrements.

Methods Employed: The [illegible] study participants are male volunteers recruited by other participants in the program. Recruits agree to return to GRC in Baltimore for 2-1/2 days of testing every 12 months (age 70 and over), 18 months (age 60-69) or 24 months (under age 60) for an indeterminate period. At entry into the program, 87% of subjects reported at least some college, 87% were identified with professional, technical or managerial occupations, 90% were presently married, 81% described themselves as financially comfortable, and of the group who returned for the fifth visit, 90% had rated their health as good or excellent on both first and fifth visits.

Testing Procedures: Psychological tests include especially designed procedures which assess verbal learning and problem-solving abilities as well as standardized tests of intelligence and personality. Physical examinations and medical histories are performed with check lists to insure completeness. Smoking habits, dietary intake, and marital and sexual adjustments are obtained by questionnaire, diary and interview. Both conventional and special procedures are used to assess physiological areas such as kidney function, pulmonary function, neuromuscular function, cardiovascular function, and special senses.

Data Management: Medical records and test results are maintained in written form in the laboratory and transferred to a data retreival and analysis system by keypunching on tabulation cards or by recording the test results directly on punched paper tape or magnetic tape. Data are maintained and used in ways which protect the privacy of participants. Sensitive material is specially encoded. Individual scientists review, evaluate and summarize the data for scientific reporting.

Major Findings: By February 28, 1974, 980 study participants had completed one or more visits to GRC for tests. They accounted for 4716 participant visits since 1958. At least four visits had been completed by 528 participants, while 200 participants had eight or more visits. There were 647 participants currently under observation, 112 had died, 84 had formally withdrawn and 137 had failed to respond to invitations to return for tests.

A systematic review has been completed for body composition, physical activity, metabolism and strength and work output measurements. Over 140 measurements and indices have been characterized with respect to cross-sectional age differences and longitudinal age changes. These results are being classified by medical status of the participants.

A summary analysis of the questionnaire, "Your Activities and Attitudes," developed by Burgess, Havighurst and Cavan has indicated a decline in social activity and a decrease in positive social attitudes in the older participants in the longitudinal study. In addition, a variety of social and demographic characteristics were isolated from this questionnaire and other records. These characteristics, such as educational attainment and marital status, showed remarkable consistency over all age groups. Only prior farm residence and protestant religious affiliation were significantly higher in our older participants.

Participants who have dropped out of the longitudinal study were compared with participants who have continued reporting for tests. Comparisons were made for the entire array of social and demographic characteristics. Participants who were here for one visit only were compared with participants who were here for two visits only and with participants who were here for three or more visits. No significant effect of leaving the study was detected for social or demographic variables.

An automated pulmonary function data processing system was developed for the purpose of improving the precision and accuracy of the data used in the longitudinal study and to explore new methods of analyzing pulmonary function tests. A main feature of the system is the maintenance of a data bank of digitized spirograms, single breath nitrogen, and nitrogen washout tracings which may be reproduced at will to prepare duplicate records for charts or referring physicians and remeasured in the future in accordance with changing methods of test analysis. The new methods of analysis currently under development use numerical integration methods to compute parameters based on the entire curve (such as mean transit time for an FEV) or compartment modeling for the nitrogen washout. The tests included in the automated system are: Slow vital capacity, forced spirogram, single breath nitrogen, open circuit nitrogen washout, and maximal ventilatory volume.

Significance to Bio-Medical Research and the Program of the Institute: A major goal of the Longitudinal Program is a deeper understanding of age-related changes in the different organ systems, and their interrelationships. The relation of functional changes in an individual to age at death, age of onset of a disease, and other end points is important for understanding aging in humans and the impact of aging on society. The intensive study of multiple variables will also provide tests of risk-factor theories for specific age-related diseases.

Proposed Course: Data collection and analyses will be continued. Continued emphasis on automation of tests, data entry, and analyses should provide improved accuracy and efficiency. Training of personnel to operate these improved systems will continue. A major summary of all aspects of this program is in progress.

Honors and Awards: None

Publications: None

Serial No. HD-CP-24
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Age Changes in Human Performance

Previous Serial Number: HD-AG4(c)

Principal Investigators: Arthur H. Norris
Alan Zuckerman
James E. Fish

Other Investigators:		
	N. W. Shock	Harold Menkes**
	A. T. Welford*	Richard Rosenthal**
	Solbert Permutt**	Warren Summer**
		Philip Norman**

Cooperating Units: Baltimore City Hospitals

Department of Psychology*
University of Adelaide
Adelaide, South Australia

The Johns Hopkins Medical Institutions**
Baltimore, Maryland

Man Years:

Total:	4.30
Professional:	2.00
Other:	2.30

Project Description:

Objectives: This project is designed to study the effects of aging on the physiological responses to and recovery from exercise--to describe age changes and to elucidate the mechanisms of these effects of aging. It is designed to identify underlying factors in the limitation of work performance and reduced mechanical efficiency in older people. For this purpose, detailed evaluation of pulmonary function and pulmonary responses to stressful agents are carried out. Other factors such as the metabolic cost of limb movement and psychomotor control of limb movement are being studied.

An additional goal is to identify and explain the role of disease and the roles of normal physiological functions and indices in the age-related limitation of work performance. Cerebrovascular, cardiovascular and pulmonary disease and functional measures such as blood pressure, reflex time, and reaction time will be considered.

Methods Employed: Measured amounts of physical work are administered to subjects of varying ages by means of a calibrated arm ergometer and quantitative mechanical analysis of limb movement. A treadmill is used to induce higher levels of work. Measurements of oxygen uptake, CO_2 elimination, pulmonary ventilation volume, heart rate, blood pressure, and electrocardiogram are made before, during and after standardized amounts of exercise. In addition, the volume of the lungs and functional capacities of the pulmonary system are evaluated. Measurements of the single breath nitrogen washout, forced expiration, specific airway conductance and static lung volumes are made by conventional methods with the subjects in the seated position. A bronchial challenge is induced with ragweed antigen and Methacholine hydrochloride (mecholyl)--a parasympathomimetic agent capable of producing changes in bronchomotor tone in humans.

Forced expiratory spirograms are analysed with respect to the mean transit time (T = the average time for air to leave the lung) and the root mean squares of the transit time ($\alpha_2^{1/2}$ = the second moment about the origin of the volume-time curve). The relation between $\alpha_2^{1/2}$ and T is used as a measure of nonuniformity of lung emptying rates.

Major Findings: 1) Age, bronchospasm and airflow patterns: In a cross-sectional study of 55 nonsmoking males ages 20 to 70, $\alpha_2^{1/2}$ increased with age whereas T did not change. A multiple regression analysis of $\alpha_2^{1/2}$ as a function of age and T yielded the following relationship $\alpha_2^{1/2} = 1.312\ (\pm 0.061)\ T + 0.0034\ (\pm 0.0009)\ \text{age} + 0.134$. Forced expiration spirograms were measured in 7 subjects between the ages of 20 and 30, before and after controlled inhalation of ragweed antigen, and in 7 age matched non-asthmatic subjects before and after inhalation of Methacholine hydrochloride. In both the antigen and mecholyl treated subjects, T increased with the onset of bronchospasm. The mean increase in T for the mecholyl treated group was 0.127 corresponding to a mean decrease in the one-second forced expiratory volume of 11.6% and a 42% mean drop in the specific airway conductance. In the antigen treated group T increased 0.428 seconds corresponding to a 32.6% mean drop in the one-second forced expiratory volume and a 57% drop in specific airway conductance. $\alpha_2^{1/2}$ was found to increase with T in both groups of test subjects. However, in no instance was the value of $\alpha_2^{1/2}$ higher than that predicted for any 20 year old with the same mean transit time. Thus, our studies show a significantly greater increase in $\alpha_2^{1/2}$ in relation to T in aging than in acute reversible bronchospasm, therby suggesting a more nonuniform pattern of lung emptying rates in age-related alteration of airflow.
2) Asthmatic responses: To date seven normal subjects and two asthmatics have been tested. Preliminary results show that for a 45% drop in specific airway conductance, the asthmatic subjects had a greater drop in the one-second forced expiratory volume than normal subjects. (Asthmatics = 20%, normals = 11.6%). This may be a reflection of a higher airway resistance with which the asthmatic subjects began the testing (Asthmatic = 3.62 ± 1, normals = 1.94 ± 0.53). Residual volume and total lung capacity increased in both groups. In the normal subjects there was no significant alteration

in the closing volume or alveolar plateau following bronchospasm. However, in the asthmatic subjects, the onset of bronchospasm resulted in a reduction of closing volume and an increase in the slope of the alveolar plateau. These findings suggest that mecholyl produces obstruction in both small peripheral and larger central airways in both groups of subjects. The elevation of the slope of the alveolar plateau in the asthmatic subjects is evidence for the existence of underlying small airway disease which results in a more nonuniform distribution of inspired gas.

3) Speed and accuracy of tapping on a single small target. Measurement of speed and accuracy of tapping on a single target (a 1-2mm dot) was used to establish the degree of dispersion of hits when no lateral movement was required between taps. In this way the aquisition of information about positioning was kept at a minimum for tapping from a height of 1-2 cm and increased for tapping on a second and third target from heights of 5-10 cm and 10-15 cm, respectively. The time for 50 taps and the maximum diameter of the scatter of hits was recorded for each target. Subjects were instructed to tap as rapidly and accurately as possible. Their strategies varied. Some attempted to achieve accuracy at the cost of increased time, others attempted to work rapidly with some sacrifice of accuracy. No attempt was made to modify the strategies chosen by the subjects. Summary of 2326 sets of targets for 638 participants in the Baltimore Longitudinal Study confirmed the relationship between speed and accuracy. There was a significant regression of target width on time required for 50 taps of approximately 9 mm per min for each of the three tapping heights used. The range of strategies used by the subjects can be inferred from the coefficients of variation of times and of dispersions of hits which ranged from 48% to 68%. Both time and dispersion increased significantly with increase in age for all tapping heights. Time for 50 dots increased by 0.0014 min per year for tapping height 1-2 cm, and by 0.0025 min per year for the 5-10 and 10-15 cm height. Dispersion of hits on target increased by 0.0294 mm per year for tapping height 1-2, 0.0228 mm per year for height 5-10 cm, and 0.0388 mm per year for height 10-15 cm. These age differences were expressed as fractions of mean times and mean dispersions for the three tapping heights. Increases in time with increase in age ranged from 0.7 to 0.9 percent per year, while increases in dispersion of hits ranged from 0.2 to 0.4 percent per year. Thus, our older participants chose strategies which protected accuracy at the cost of increased time to perform the task.

Significance to Bio-Medical Research and the Program of the Institute:

The pathophysiology of airway obstruction comprises a wide variety of anatomic and physiologic alterations. Because of the limitations of current methods of study, most of the physiological alterations are poorly if partially understood. Measuring the distribution of transit during forced expiration provides a measure of the degree of uniformity of expiratory flow, and therefore is a valuable tool in understanding the pattern of airflow obstruction in diseases which have limination of flow as their main component. In allergic bronchospasm it has been proposed that the release of histamine results in afferent vagal stimulation leading to reflex bronchoconstriction, and that asthmatic individuals have a "hypersensitive" response to these stimuli. The present study is significant in that it demonstrates not only a difference in the degree of the bronchospastic response, but also a difference in the pattern of airway obstruction.

The decline of the ability of some older people to perform their day-to-day activities and to engage in pursuits which contribute to the economic and social strength of our society represents a national loss. Identification of the physiological, medical and social correlates of high levels of physical strength and psycho-motor performance in middle and old age, as well as declines in these abilities, should lead to techniques designed to reduce the rate of decline in performance capacities with age.

Proposed Course: Additional numbers of asthmatic subjects are needed to evaluate the significance of the preliminary findings. In an attempt to define the role of the autonomic nervous system as a mediator of allergic bronchospasm, these same physiologic parameters will be monitored in asthmatic subjects following bronchial challenge with ragweed antigen extract, with and without the influence of effective vagal interference using atropine.

Measurements of muscle strength in maximum power generating ability during arm exercise will be continued. Cardiovascular, ventilatory and metabolic responses to standardized arm ergometer exercise and monitored treadmill exercise will be used to classify participants into fitness categories. Measurements of lung volumes and uniformity of pulmonary ventilation will be made to characterize the respiratory competence of the longitudinal studies participants and to evaluate the benefits of the cessation of smoking in this group.

Honors and Awards: None

Publications:

Norris, A. H. and Shock, N.W.: Exercise in the adult years. Chapter 26 in Johnson, W.R., and Buskirk, E.R.. (eds.): Science and Medicine of Exercise and Sport (2nd edition) New York, N.Y. Harper & Row, 1973, pp. 346-362.

Serial No. HD-CP-25
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Age Relationships of Body Composition, Nutrition and Physical Activity

Previous Serial Number: HD-AG5(c)

Principal Investigators: Arthur H. Norris
Reubin Andres

Other Investigators: N. W. Shock
Marlene Butler
Janet Kusterer
Paul J. Davis*

Cooperation Units: Department of Medicine*
Baltimore City Hospitals

Professor George Vose, Research Institute
Texas Woman's University, Denton, Texas

Man Years:

Total:	1.95
Professional:	.45
Other:	1.50

Project Description:

Objectives: This project is designed to describe age differences and age changes in body composition, nutrition, and physical activity. Mechanisms of interaction of these functions and behaviors will be sought. The relationship of these measurements to other physiological, psychological and biochemical variables will be examined.

Methods Employed: Height, weight, and body circumferences of longitudinal study participants are obtained by standard anthropometric methods. Roentgenographic and anthropometric estimates of skeletal mass are combined with height, weight, and body circumferences to provide an estimate of body fat. Other estimates of fat include skinfold thickness measurements and fat thickness measurements from X-rays. Indices of lean body mass include: (1) basal metabolic rate determinations, (2) twenty-four hour urinary excretion of creatinine, and (3) total body water and extracellular water determinations by indicator dilution. Nutrient intakes and activity calories are estimated from a diary and a self-administered questionnaire. All such measurements are repeated in the course of each subject's participation in the longitudinal program.

Major Findings: Analysis of the dietary intake data indicated that one third of the participants in the longitudinal studies were, on their own initiative, taking a vitamin supplement containing thiamin, riboflavin, niacin and vitamin B_6. A larger proportion (45%) of the men 60-79 years old were taking a supplement. In other age groups only 25% took a vitamin supplement.

Dietary intakes of these B vitamins were the same for the vitamin and non-vitamin takers in all age groups. Mean thiamin, riboflavin, and niacin dietary intakes met or exceeded the NRC recommended dietary allowances. The mean vitamin B_6 intake of 1.65 mg was only 80% of the amount recommended. In men taking supplementary vitamins, plasma pyridoxal phosphate (PLP) levels were higher (20.5 ng/ml) than for those not taking a supplement (12.3 ng/ml). Likewise, urinary excretion levels of thiamin and riboflavin were significantly higher in those taking vitamin supplements. These results increase our confidence in the reliability of the independently-obtained historical and laboratory data.

Total physical activity was estimated from a self-administered questionnaire. Results for 758 men showed a decline in activity from 1250 Cal/day in participants 25-44 years old to 870 Cal/day in participants 75 years of age and older. Participants were divided into groups of exercisers and non-exercisers. Exercisers were defined as subjects who performed work valued at .06 Cal/kg body weight/minute for 15 minutes or more per day on the average. Situps, golf, swimming and bike riding are examples of .06 Cal/kg/min activities. Distributions of activity calories for exercisers and non-exercisers were nearly identical for every age decade group between 25 and 84 years of age. Thus, comparisons of activity calories to body composition or nutritional intake measurements were not influenced by exercise category. Activity calories were positively correlated with intracellular water, body weight, lean biceps circumference, and an index of muscle mass from the Behnke anthropometric index ($r = 0.268$ to .415 independent of age).

Specific sports activities (such as golf, tennis, bowling and swimming) were examined. The percent of older subjects participating in these activities was sharply lower than the percentage of younger subjects participating. For example, 7% of older subjects bowled as compared with 25% of younger subjects. On the other hand, 32% of these men performed situps in both old and young age groups. This suggest that sociability or "having a partner" contributes to the maintenance of some physical activites in older people.

Significance to Bio-Medical Research and the Program of the Institute: Nutritional deficiencies in the aged are known to be common and are generally attributed more to the socio-economic deprivation of this group than to biological or physiological aging effects. The volunteers in the Longitudinal Study Group are not a deprived group--it may be characterized as upper-middle class and has a very high educational level. It, therefore, offers a unique opportunity to study nutritional status under very favorable conditions. The nutritional effects of biological aging per se may therefore be separated from what might be called "social aging."

Certain age changes in organ systems and various diseases are thought to be affected by diet, level of physical activity, and body composition. From the repeated assessment of these factors over time, it may be possible to determine their relative contributions to longevity and the maintenance of health and vigor in later life. Difficulties associated with obtaining retrospective estimates of eating habits, activity and body composition in the past make a prospective approach necessary for the collection of reliable information.

Proposed Course: Studies of diet, physical activity and body composition will continue. Data already collected will be further analyzed. Interactions of changes in body composition, food intake, food composition, kind and amount of physical activity, disease, and age will be examined. Specifically, body fat and lean body mass estimates, nutrient intakes and physical activity category will be used in an analysis of risk of cardiovascular disease.

Honors and Awards: None

Publications: None

Serial No. HD-CP-22
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Marital, Sexual and Social Factors in Aging

Previous Serial Number: HD-AG1(c)7

Principal Investigator: C. Martin

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	1.00
Professional:	1.00
Other:	.00

Project Description:

Objectives: Major goals are to: (1) compare longitudinal subjects of diverse age for evidence of marital, sexual and social variables that vary in relation to age, (2) determine whether individual differences in sexual functioning may be related to social, physiologic or psychologic characteristics, and to (3) discover whether any aspect of prior marital, sexual or social experience may be associated with entities of disease or longevity.

Methods Employed: Each subject, on one or another visit to GRC, is asked to contribute an interview dealing with his history of marriage and sexual activity. In making this request, the investigator outlines the objectives of the study, provides assurance of confidence, and emphasizes the voluntary nature of such a contribution. All questions are memorized as well as the response categories needed for recording replies. Chapters of the life history pertaining to residential, military, religious, educational, occupational and parental-home experience are reviewed before introducing questions relating to marital adjustment and sexual conduct. This initial questioning provides information not otherwise obtained, aids rapport, and establishes various reference ages against which previous behaviors can be oriented as to time. By now, 632 subjects have completed interviews, another 11 have partly completed interviews, while 14 declined interviews--a refusal rate of 2.1 percent. These data are available in the computer master file.

Major Findings: Inquiry with respect to frequencies of sexual activity in the recent past reveals that after an initial plateau, average frequencies of sexual activity show a steady decline with advancing age. New data, shown below, are of interest because they are subject to recent recall, include both coital and non-coital forms of sex expression, and compare favorably with similar data reported in Sexual Behavior in the Human Male, by Kinsey, Pomeroy and Martin.

Median Frequencies of Sexual Activity Per Week

Age Interval	Present Study	Kinsey Study	Age Interval	Present Study	Kinsey Study
20-24	1.97	2.10	50-54	1.00	0.90
25-29	2.16	2.24	55-59	0.68	0.73
30-34	2.33	1.91	60-64	0.67	0.52
35-39	1.75	1.73	65-69	0.30	0.30
40-44	1.60	1.41	70-74	0.42	----
45-49	1.12	1.10	75-79	0.26	----

Although the several studies were conducted some 25 years apart in time and involve differences in methods of data collection, analysis and sample composition, the evident similarity of findings is indicative of a pattern of sexual aging in the male that is relatively unaffected by social change. It is also of interest that inquiry as to whether respondents would seek treatment for a restoration of sexual vigor, if this were possible, revealed no gradient in affirmative replies by age. In fact, about two-thirds of subjects aged 40 to 79 clearly expressed their disinterest in such a possibility under present circumstances. The latter finding is seen as reflecting a high level of acceptance of the effects of aging on sexual functioning.

Significance to Bio-Medical Research and the Program of the Institute: Research inquiry into factors associated with marriage and sexual functioning, as possible precursors of chronic disease, has been almost non-existent. Publication will keep alert others to the existency of the study and display the character of the data obtained. Moreover, the detail on methods and the demonstration that specific behavioral variables vary according to age will be of interest to others engaged in life history research.

Proposed Course: Analyses will be undertaken to relate measures of quantity of sexual activity and status of coital potency to various social, physiologic and other variables as programming time becomes available. With reference to the third objective, once diagnostic classifications on coronary disease and prostatic health become available, it will be possible to employ these in tandem with data on blood pressure, serum cholesterol and other indicators of pathology, to learn whether subjects with abnormal values may differ from normal subjects with respect to various kinds of marital, sexual and social experience.

Honors and Awards:

Dr. Martin discussed "Marital and Sexual Factors in Relation to Age and Health" before the Psychosomatic Conference, The Johns Hopkins School of Medicine, January 15, 1974.

Publications:

Martin, C.E.: Marital and sexual factors in relation to age, disease and longevity. In: Wirt, R.D., Winokur, G. and Roff, M. (eds.): Life History Research in Psychopathology, Volume IV, Minneapolis, Minn., University of Minnesota Press (in press).

Serial No. HD-CP-13
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Dermatoglyphics and Clinical Medicine

Previous Serial Number: Same

Principal Investigator: Chris C. Plato

Other Investigators:
W. Wertelecki*
J. D. Niswander**
F. S. Steinberg***
J. Cereghino#
C. C. Fraumeni, Jr.##
H. Fessas###

Cooperating Units:

Department of Pediatrics*
Medical University of South Carolina

Department of Human Genetics, NIDR**

Program Statistics Branch, NICHD***

C & F Research, NINDS#

Epidemiology Branch, NCI##

Nicosia, Cyprus###

Man Years:

Total: .80
Professional: .50
Other: .30

Project Description:

Objectives: A. To establish possible statistical associations between various clinical anomalies and dermatoglyphic features; B. to establish suitable normal control panels, Caucasian and Negro and male and female, which can be used in future studies to determine associations between dermatoglyphics and various diseases. Also data are being collected from the Gerontology Research Center's longitudinal subjects to find if there are differences in the dermatoglyphic frequencies among individuals of different age groups. The possibility of differences in the haptoglobin frequencies in different age groups is also being investigated.

Methods Employed: Finger and palmprints were collected from patients with cleft palate, congenital heart disease and leukemia, and from their parents and sibs where possible. Prints were also obtained from patients with Down's Syndrome and other types of mental retardation. A study was also initiated to collect prints from children with Thalassemia (Cooley's anemia) and their first degree relatives. This is of special importance since the heterozygote parents and sibs, as well as normal sibs, are clinically identifiable. Prints taken from sibs are to serve as internal controls in addition to the panels of white and black Americans used as population controls. Several methodologies were developed to facilitate detailed comparisons.

Major Findings: Statistical analysis revealed significant relationships between the disease entities of leukemia, Down's Syndrome and deletion of the long arm of the 18th chromosome, and certain dermatoglyphic characteristics. Of special interest were the investigations of palmar creases which we reclassified. Leukemia and Down's Syndrome were both shown to be associated with simian and sydney lines. We also described the "thenar" line which we showed to be genetically determined.

Significance to Bio-Medical Research and the Program of the Institute: The finding of significant statistical associations between various diseases and dermatoglyphic markers may assist in the determination of their etiology.

Proposed Course: To continue this method of investigation taking into consideration other clinical anomalies.

Honors and Awards:

Mr. Plato presented a report on Down's Syndrome to the 8th Joint Meeting of the U.S.P.H.S. Professional Associations, in Phoenix, Arizona.

Mr. Plato lectured at the Department of Pediatrics, University of South Carolina, Moore Clinic, Johns Hopkins and the Cyprus Medical Society.

Mr. Plato presented two invited papers at the Dermatoglyphics Section of the IV International Congress of Anthropological and Ethnological Sciences, Toronto, Canada, and Chaired the session of Clinical Dermatoglyphics. Reports will appear in the proceedings.

Publications:

Wertelecki, W., Plato, C.C., Fraumeni, C., and Niswander, J.: Dermatoglyphics in leukemia. Pediatric Research 7:620-626, 1973.

Wertelecki, W. W. and Plato, C.C.: The distribution of palmar creases in various diseases and random populations. In: Proceedings of the L. S. Penrose Memorial Colloquium. Stringer Press, Berlin.

Steinberg, F.S., Cereghino, J. and Plato, C.C.: The dermatoglyphics of American negroes: With a review of the negro dermatoglyphics. Special issue of the American J. Physical Anthropology (in press).

Serial No. HD CP 1a
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland 2

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Dermatoglyphics of Primitive and Other Populations

Previous Serial Number: Same

Principal Investigator: Chris C. Plato

Other Investigators:
D. Carleton Gajdusek*
Ralph Garruto**
Robert MacLennan#
H. Brown***
Marshall T. Newman##

Cooperating Units:

C & F Research, NINDS*

Special Chronic Disease Studies**
Department of Anthropology
Pennsylvania State University

International Agency for Research on Cancer#
WHO, Lyon, France

Department of Anthropology##
University of Washington
Seattle, Washington

Port Moresby, New Guinea***

Man Years:

Total:	.50
Professional:	.30
Other:	.20

Project Description:

Objectives: This project represents part of a multidisciplinary effort in conjunction with the World Health Organization and other institutions to study various genetic, clinical and anthropological markers among the developing peoples of the South Pacific and South America. Our part is to establish the distribution patterns of the dermatoglyphic features among the various populations and geographical areas, and map them in such a way as to serve as reference and source of comparison.

Methods Employed: Dermatoglyphics were collected by the Faurot inkless method. Data were evaluated. Our main area of study is South Pacific and Australasia in general, Peru, Colombia, and Mexico.

Major Findings: Dermatoglyphic characteristics are for the most part governed by multiple genes and complex types of inheritance. Consequently, they are not readily vulnerable to the influences of selection or genetic drift due to isolation or migration. Thus, the incidence of dermatoglyphic characteristics found in different populations may reveal the nature of the relationships existing between populations in the remote as well as the more recent past.

In Cyprus, similarities found with respect to dermatoglyphics and to thalassemia between peoples in the highlands and the lowlands are indicative of an ancient relationship; wide differences in blood groups suggest recent changes resulting from isolation and genetic drift among highland peoples, and from migration and admixture among the lowland people. Extensive reviews were completed for the Australasian populations (we contributed most of the publications for this region), Caucasians, Negroes and the Amerindians. We also completed an atlas of the world wide distribution of the dermatoglyphic features. All of these are in press.

In New Guinea, the Fore (a people affected with Kuru) had different dermatoglyphics and also blood groups than their neighbors, the Anga. The findings indicate ancient and persistent genetic differences due to mechanisms affecting isolation. Among the Quechuas of Peru, dermatoglyphic differences were evident while serological factors were quite alike. In contrast to the Cyprus situation, this latter finding is suggestive of ancient genetic differences which have largely disappeared because of more recent admixture.

Significance to Bio-Medical Research and the Program of the Institute: The thorough evaluation of genetic and clinical markers of the few remaining primitive populations will allow scientists at a later time to ascertain the effects of "western" culture, infections and environmental pollution upon populations, thus far relatively unexposed to these kinds of influence.

Honors and Awards:

Mr. Plato delivered two invited reports at the L. S. Penrose Memorial Colloquium in Berlin, and was nominated treasurer of the International Dermatoglyphic Association.

Publications:

Plato, C.C.: Variation and distribution of the dermatoglyphic features in different populations: An atlas of dermatoglyphics. In Hirsch, W. (ed.): Proceedings of the L. S. Penrose Memorial Colloquium. Stringer Press, Berlin.

Plato, C.C., Gajdusek, D.C. and MacLennan, R.: The dermatoglyphics of the peoples of New Guinea: A review. In Mavalwala, J. (ed.): Dermatoglyphics: An International Perspective. The Hague, Mouton Press. (in press)

Plato, C.C., Garruto, R.M., Hoff, C. and Baker, T.: Genetic studies among the Quechua Indians of the Peruvian Highlands. I. Digital and palmar dermatoglyphic patterns among southern Peruvian Quechua. *Human Biology* (in press).

Plato, C.C., Brown, H.A. and Gdjdusek, D.C.: The dermatoglyphics of the Elema peoples from the gulf district of Papua New Guinea. Special issue of the *Am. J. Physical Anthrop.* in honor of H. Cummins (in press).

Plato, C.C. and MacLennan, R.: The dermatoglyphics of the Maprik subdistrict of the Sepik district of New Guinea. *Zeits. fur Morph. und Anthrop.*, Stutgart (in press).

Serial No. HD-CP-14
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Twin Studies: I. Structure and Color of the Iris
II. X Chromosome Inactivation
III. Dermatoglyphics and Zygocity Determination

Previous Serial Number: Same

Principal Investigator: Chris C. Plato

Other Investigators: J. T. Schwartz*
W. Wertelecki**
J. D. Niswander#

Cooperating Units: F & D Research, NEI*

Department of Pediatrics**
Medical University of South Carolina

Department of Human Genetics, NIDR#

Man Years:

Total:	.20
Professional:	.20
Other:	.00

Project Description:

Objectives: 1. To determine the degree of variability and heritability of the structure of the human iris. 2. To test the Lyon Hypothesis regarding X chromosome inactivation. 3. To develop, through dermatoglyphics, further criteria for twin zygocity diagnosis.

Methods Employed: Thorough eye examinations, color slides of the irises and finger and palm prints were obtained from over 600 pairs of twins. Zygocity was established by elaborate blood group typing, perinatal information, and physical similarities. In addition to the twins we are also including in this study 250 pairs of sibs to serve as controls. Fourteen pairs diagnosed as monozygotic but being discordant for the Xg^a sex linked blood group were involved with their parents and sibs, in the X chromosome inactivation study.

Major Findings: Through our twin studies we demonstrated for the first time that mother-fetus incompatibility, due to the Xg^a blood group, may result in fetal loss. Because of the sex-linked inheritance of the Xg^a, the fetal loss is confined only to the female fetus. The twin data confirmed our findings from the leukemia studies that the simian and sydney palmar creases are familial and the "thenar" line which we described is inherited.

Significance to Bio-Medical Research and the Program of the Institute: 1. To establish possible associations between the iris structure and eye disorder; 2. to provide supporting or rebutting evidence for the Lyon Hypothesis for X chromosome inactivation; 3. to offer additional means for determining twin zygocity; and, 4. to establish hereditary patterns in the transmission of the dermatoglyphic features.

Proposed Course: To evaluate the data already collected and to perform the appropriate statistical tests.

Honors and Awards:

Mr. Plato has been invited to present reports at the I International Congress of Twin Studies in Rome, Italy.

Publications: None

Serial No. HD-CP-16
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Genetic Studies on Longitudinal Program Subjects

Previous Serial Number: Same

Principal Investigator: Chris C. Plato

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	00
Professional:	00
Other:	00

Project Description: This project has been made part of HD-CP-13, "Dermatoglyphics and Clinical Medicine."

NICHD ANNUAL REPORT
July 1, 1973 through June 30, 1974
Gerontology Research Center
Laboratory of Molecular Aging

The research program on the control of intermediary metabolism focuses on the mechanisms by which metabolic processes are regulated. The thrust of the work is directed towards what we conceive may be the "next frontier" in biomedical research — membrane biology. The problems and questions studied relate to: (1) the molecular organization of the membrane; (2) the role of the membrane in vectorial transport of small molecular species and protonmotive force; (3) the membranal reception of hormonal stimuli, the transduction and propagation of these stimuli; (4) the catalytic and integrative function of membranes; (5) the turnover of membranes; and (6) the failure to maintain membrane structure, leading to cell death. The fundamental information developed by these studies is essential to and is applicable to the understanding of the biological processes whereby physiological control systems become modified during development and aging.

The uptake of D-glucose into vesicles of membranes isolated from the apical region of the plasma membrane of the renal cortical proximal tubule was shown to be a Na^+-gradient dependent facilitated transport process. In the brush border system Na^+ gradients were artificially imposed, whereas in more intact systems it is the ouabain sensitive active extrusion of Na^+ across the lateral and basal membranes of the tubule cell that presumably establishes the gradient required for uphill sugar transport. The initial rate of D-glucose transport into the membrane vesicles was stimulated 700% by the Na^+ gradient. The $K_{(stimulation)}$ was 30 mM Na^+. The stimulated uptake was the consequence of a reduction in apparent Km for the sugar and an increase in Vmax. The uptake of D-glucose in the presence of an initial Na^+ gradient showed an overshoot above the equilibrium value with ultimate return to the equilibrium value as the Na^+ gradient was dissipated during the incubation. Neither Li^+, K^+ nor choline ions caused augmentation of transport. The patterns of inhibition of the Na^+ stimulated transport of D-glucose by sugar analogs and phloridzin closely resembled known physiological data. These findings are consistent with and provide the first strong experimental support in renal membrane systems for the Na^+ gradient uphill transport of D-glucose hypothesized by Crane.

The renal brush border membrane proved to be an effective model for the study of amino acid transport in the kidney. For the first time, the uptake of an amino acid (alanine) by the isolated membrane was shown to be consistent with a concept for a Na^+ gradient dependent facilitated transport of amino acids across the luminal membrane. Na^+ also was found to stimulate γ-glutamyl transpeptidase. This enzyme, whose specific activity was found to be enriched manyfold in the purified brush border preparation, relative to the cortex homogenate, has been proposed recently as the amino acid carrier.

Investigations of hormonal regulation of metabolism were focused on the mechanisms of endocrine control of renal transport. Specific questions dealt with: (1) the localization in the renal cortex of the membrane bound hormonally stimulated adenyl cyclase; (2) the binding of cAMP and cGMP to luminal membranes; (3) the hydrolysis of cyclic nucleotides by membranal phosphodiesterase; (4) the identification and characterization of the membrane bound protein kinase; (5) the identification of the endogenous protein in the membrane that is phosphorylated; and (6) how the presumed phosphorylated-dephosphorylated protein acts to control the transports of Pi, Ca^{2+} and, perhaps Na^+.

The specific activities of renal cortical adenyl cyclase, basal as well as that activated by NaF, catecholamines and parathyroid hormone, were not elevated significantly in the brush border membrane relative to those in other membranes. This finding suggests that in the renal cortex cAMP is generated on the anti-luminal regions of the plasma membrane. However, since cAMP must traverse the cell to interact with the luminal membrane, e.g., in the action of parathyroid hormone on Pi transport, and in the appearance of cAMP in the urine, the binding of cAMP to the brush border membrane was characterized. Binding was saturable, approximately 1 pmole/mg protein was bound at 20 nM cAMP, with multiple binding sites. Binding was reversible. Bound cAMP was resistant to hydrolysis by endogenous or exogenous phosphodiesterase. Binding was unaffected by hormones, pH and Na^+ gradients, but was markedly inhibited by sulfhydryl reagents (partially reversed by thiols). cGMP was bound at a rate about 0.1 that of cAMP. cIMP, dibutyryl cAMP and cGMP inhibited cAMP binding.

The levels of cyclic nucleotides at the luminal membrane region of the tubular cell could be controlled by brush border phosphodiesterases. Enzymes hydrolyzing both cAMP and cGMP were evident. At least two enzymes acting on cAMP were found. Phosphodiesterase I was maximally active at pH 8, stable at 53°, apparent Km for cAMP $>10^{-3}$ M, and, upon EGTA inhibition, reactivated by Ca^{2+} but not by Mg^{2+}. Phosphodiesterase II was maximally active at pH 6-7, labile at 53°, and, upon EDTA inhibition, reactivated by Mg^{2+} but not Ca^{2+}. Cobalt activated both chelator-inhibited enzymes.

Protein kinase activity was demonstrated in which the brush border membrane acted as both enzyme and substrate. The apparent Km for ATP was 62 μM and activity was Mg^{2+} dependent (10 mM optimal). The ability of the membrane to phosphorylate itself may be important in control of proximal tubular function.

The role of the membrane in the control of mitochondrial oxidations and, indeed, of the basic mechanisms of energy transduction was studied. These investigations were centered on the control of the complete oxidation of pyruvate, a process involving the operation of both pyruvate dehydrogenase and the entire tricarboxylate cycle, and involved the two main approaches to the regulation of intermediary metabolism: (1) the study of enzyme kinetics in vitro; and (2) the variations in the steady state concentrations of key intermediates upon altering the flux through the metabolic pathway.

To pinpoint which of the several reactions of the tricarboxylate cycle is the locus of control in blowfly flight muscle, the steady state concentrations of citrate, isocitrate, α-ketoglutarate, malate, CoA, acetyl CoA and succinyl CoA were measured in mitochondria during the transition from State 4 (respiration limited by ADP) to State 3 (fully active). On activating respiration with ADP, the mitochondrial contents of citrate, isocitrate and acetyl CoA decreased whereas those of α-ketoglutarate, succinyl CoA, malate and CoA increased. Measurements of the tri- and dicarboxylates in the flight muscle of blowflies in vivo before and after initiation of flight were entirely parallel to those found on the activation of mitochondrial metabolism in vitro. These changes, combined with the knowledge that the reaction catalyzed by isocitrate dehydrogenase is not at equilibrium, allowed the unequivocal identification of this enzyme as the locus of control in this tissue.

It was found previously that the activity of isocitrate dehydrogenase was inhibited by Ca^{2+}. Thus, Ca^{2+} could effectively function to control the complete oxidation of pyruvate via the tricarboxylate cycle. A kinetic study was carried out on the inhibition of pyruvate oxidation by Ca^{2+}. Pyruvate oxidation was inhibited almost completely by physiological levels of Ca^{2+}; the Ki was 10 µM. The kinetic nature of the inhibition with respect to components of the system, including ADP, Pi and pyruvate, was determined.

The control of pyruvate dehydrogenase activity in isolated rabbit heart mitochondria by a specific kinase and phosphatase, which interconvert phosphorylated (inactive) and dephosphorylated (active) forms of the enzyme, was demonstrated. Dehydrogenase activity was decreased when intact mitochondria were incubated with ATP, oligomycin and NaF. This decrease in dehydrogenase activity was correlated with the incorporation of $[\gamma\text{-}^{32}P]$ATP into mitochondrial protein and a decrement in the oxidation of pyruvate via the tricarboxylate cycle. Kinase activity was inhibited by pyruvate and Pi and the kinetics of these inhibitions were determined. Inactivation of the dehydrogenase was enhanced by divalent cation ionophores. The inactive phosphorylated dehydrogenase was completely activated by dephosphorylation. The phosphatase was inhibited by NaF and EDTA, and was activated by Mg^{2+}. Reactivation of the dehydrogenase was extremely sensitive to Ca^{2+}, only 10^{-6} to 10^{-7} M was needed for full activity.

Steady state concentrations of intramitochondrial NAD and NADH in flight muscle were measured in order to determine whether the transfer of electrons to the respiratory chain was rate-limiting relative to the transfer of electrons from the respiratory chain to O_2. Intramitochondrial NADH was found to rise on adding ADP to mitochondria oxidizing pyruvate. Experiments in which NAD and NADH were isolated from flight muscle showed that there was a parallel rise in NADH content on initiation of flight. Thus, these results obtained both from the intact insect, in vivo, and from isolated mitochondria, in vitro, tend to indicate that the activity of the dehydrogenases increase relative to that of electron transport and phosphorylation, on imposition of an energy demand upon the mitochondria. This concept is completely contrary to all other hypotheses on mitochondrial bioenergetics and will be investigated further.

The research program on the control of the expression of genetic information focuses on the interactions of the molecules that are concerned in the propagation of genetic information. The interactions of DNA and RNA with each other, with proteins, and with metal ions are studied with the object of understanding these interactions in terms of biological function and particularly in the replication of DNA, the transcription of RNA, and the synthesis of protein, are explored. Synthetic analogs of nucleic acids are used to probe the nature of viral infection and its changes with cellular aging, and to determine how the uptake of foreign macromolecules by cells is affected by cellular aging; it is hoped in this way to produce pharmaceutical agents to offset deleterious age changes. Studies are carried out to elucidate the biological role of important substances like hemoglobin and how structural changes in these substances can lead to aging impairment.

The interaction of metal ions with nucleic acids is of importance because of the requirement of a variety of metal ions in their biological activity and also because of the deleterious influence that environmentally introduced metals can have on nucleic acid structure. This year we employed metal ions for a third purpose: to serve as a probe of the mechanism of transcription of RNA polymerase. We had previously demonstrated the remarkably different way in which *cis* and *trans* $Pt(NH_3)_2Cl_2$ react with DNA. The difference in the behavior of these geometrical isomers of a platinum complex was used as a novel means of modifying the DNA template by complex formation involving the different geometries. These experiments lead to the conclusion that the *cis* Pt complex at low concentration and the *trans* complex at higher concentration decreased the length of the message units produced, with the positions of the Pt atoms serving as the message limiting loci. The geometry of the *trans* complex prevented its interaction with more than one base of the same strand, but the *cis* complex could readily interact with two adjacent bases, thus restricting their rotation. It appears therefore that RNA polymerase activity stopped whenever such a geometrically restricted base sequence was reached.

In an effort to understand how the binding of proteins to DNA in chromatin depends on the amino acid sequence of the proteins, we studied the interaction of model polypeptides of lysine-rich histones with DNA, and found that different amino acids affected the binding of lysine to the phosphate groups of DNA in different ways. Alanine had very little effect on this binding, whereas tyrosine and phenylalanine had important effects. The placement of amino acid residues between lysines altered the geometry of interactions between lysine and DNA phosphate. The binding of all of these polypeptides to DNA was modified by metal ions.

Through the use of difference ORD spectrophotometry it was shown that poly U message induced a conformational change in the structure of *E. coli* ribosomes. The addition of transfer RNA to the ribosome-message complex had little effect on the rotatory properties.

The thermally induced conformational transitions of single stranded stacked polynucleotides were non-cooperative; *i.e.*, the ordered structure of the molecule broke down gradually. Copper(II) ions made these transitions highly cooperative; *i.e.*, once the ordered structure had been distorted anywhere, it broke down everywhere at once.

It was established that beef pancreatic deoxyribonuclease I, the most frequently used enzyme for splitting DNA, generally split the phosphodiester bond on only one strand adjacent to a given base pair, regardless of the metal ion used to activate the enzyme. These results are in contradiction to the findings from the previously used more indirect methods for studying DNA cleavage.

Initial studies of the effects of metals on WI-38 cells grown in tissue culture indicated that the concentration of some metal ions was higher in "old" cells than in "young".

Aged cells have decreased ability to protect themselves actively (through the formation of new protein) against viruses; nevertheless, these cells effectively supported the synthesis of viral proteins. During the past year, in an attempt to locate the defects in aged cells we investigated two cellular systems: (1) membrane, for its ability to accept or reject macromolecular material from media; (2) cytoplasm, for production of errors in viral proteins. These aging changes were not sufficient to explain the loss of efficiency of the cells.

The study of vinyl analogs of polynucleotides led to the finding that these substances produce selective toxicity against viral leukemia. To date, this selectivity was detected, in various degrees, in the following systems: (1) Analogs had only a low toxicity to cells in culture and to mice and protected both of them against infection by murine leukemia virus. On the other hand, cells in culture were not protected against several other non-oncogenic viruses. (2) Analogs inhibited rather strongly the reverse transcriptase activity which is characteristic of oncogenic viruses. A detailed investigation of this inhibition revealed that usually only analog-template interactions occurred, but in one single combination of polyadenylic acid with polyvinyladenine the ternary interaction — enzyme-analog-template — was proven.

It was demonstrated that hemoglobin-bound copper directly oxidized the iron in hemoglobin in the absence of oxygen. The copper binding site on the hemoglobin molecule bound other metal ions as well as copper, although only copper produced oxidation. The common binding site for these metals was suggested by competitive binding experiments and a reduction in the rate of copper induced oxidation by the other metals. A possible regulatory role of this metal binding site was suggested by a three-fold increase in oxygen affinity when 1 zinc/2 hemes was present.

Serial No. HD-GMA3
1. Gerontology Research Center
2. Laboratory of Molecular Aging
3. Molecular Chemistry Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Structure of Nucleoprotein, Ribosome, and Interactions of Components Related to Protein Synthesis

Previous Serial Number: Same

Principal Investigator: G. Eichhorn
J. Butzow
Y. Shin

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	1.2
Professional:	1.0
Others:	0.2

Project Description:

Objectives: (1) To determine the conditions for the binding of proteins to nucleic acids, (2) to examine the mediation of this binding by metal ions, (3) to elucidate the structure of chromatin with the help of these model systems, (4) to understand how cellular differentiation and aging may be related to nucleoprotein structure, (5) to study the structure of the ribosome and its interaction with other substances engaged in protein synthesis.

Methods Employed: (1) The interactions of proteins and nucleic acids, and the effect of metal ions on these interactions are studied by optical rotatory dispersion, circular dichroism, spectrophotometry, to determine conformational changes, and by infrared and nmr techniques, to determine binding sites. (2) Models containing selected characteristics of chromatin are examined to determine which characteristics are responsible for chromatin function. (3) Conformational changes involved in the interaction of ribosomes, messenger RNA, transfer RNA, and other factors involved in protein synthesis are studied by means of optical rotatory dispersion and fluorescence.

Major Findings: A. Specific effects of various amino acids on binding of histone-like polypeptides to DNA. In an effort to understand how the binding of proteins to DNA in chromatin depends on the amino acid sequence of the proteins, we have studied the interaction of model polypeptides of lysine-rich histones with DNA, and found that different amino acids affect the binding of lysine to the phosphate groups of DNA in different ways. Alanine has very little effect on this binding, whereas tyrosine and phenylalanine have important effects. The placement of amino acid residues between lysines alters the geometry of interactions between lysine and DNA phosphate. The binding of all of these polypeptides to DNA is modified by metal ions.

B. A new method for studying the stereochemical fit between DNA and protein. It has been generally known that different conformations of DNA – protein complexes can be obtained by mixing the components directly, or by a gradient dialysis technique, in which the components are slowly permitted to come together by changing salt concentrations. We have begun to compare the systems under which direct mixing is feasible with those in which it is not. Histone I, for example, can be directly mixed with DNA, but polylysine, poly(alanine·lysine) cannot; neither can polylysine and poly d(AT). These differences can be explained if the lysines in polylysine and other polypeptides can bind in many ways to the DNA, and the initial binding produced on direct mixing is not necessarily the thermodynamically most stable binding. Histone I has its lysine residues separated in such a way that the spontaneous binding sites are also the equilibrium binding sites. If this explanation is correct, a comparison of the two mixing techniques should establish whether or not the equilibrium complex can be spontaneously produced.

C. Copper induces poly d(AT)·polylysine structure in DNA-polylysine. We have previously reported a complex between DNA and polylysine with a unique rotatory dispersion, produced when the components of the complex are heated with copper(II). It has now been observed that this same rotatory dispersion is produced by poly d(AT) in the absence of copper. Since copper binds more strongly to the GC rich regions of DNA, and polylysine binds more strongly to the AT rich region, the similarity of the two rotatory dispersions is explained by the copper ions binding to the GC rich regions of DNA, thus preventing these regions from binding polylysine. The latter then attach themselves only to the AT rich regions of DNA, thus accounting for the d(AT) like conformation. Thus copper - polylysine could prove a useful reagent in determining which DNA regions in chromatin are bound to protein.

D. Physical chemical studies on the interaction of ribosome, messenger RNA, and transfer RNA. Direct-difference ORD measurements have been used to detect small differences in conformation in a system involving the *E.coli* 70S ribosome. We have ascertained a significant difference between the system ribosome·poly U and the separate components. No significant additional difference was found on adding either charged or uncharged phenylalanyl-t-RNA to this system. Thus it appears that poly(U) message induces in the structure of the ribosome a conformational change that is not enhanced significantly by t-RNA.

Significance to Bio-medical Research and the Program of the Institute: An understanding of the structure of chromatin is required to understand the mechanism of gene regulation, which is of direct consequence to the study of development and aging. The ribosome is the locus of protein biosynthesis, and the agency or locus of much of its control. Our physical studies are designed to provide direct information about structures and interactions which are intimately involved in the synthesis process and its ribosomal control. If we are able to understand these interactions, it will then be possible to determine how they may change with age.

Proposed Course of Project: The interactions of polypeptides with DNA, and the effect of metal ions on these interactions, will be continued in an effort to understand not only the binding of protein to DNA in chromatin, but also protein-DNA interaction as in the binding of nucleic acid enzymes to DNA. The direct mising and gradient dialysis techniques for the formation of DNA-protein complexes will be used to determine which proteins spontaneously form equilibrium complexes with DNA. The copper-polylysine reagent will be further investigated for feasibility in probing the structure of chromatin. Having demonstrated conformational change in the ribosome using ORD techniques we shall now try to determine at what stages during the translation process such conformational changes occur.

Honors and Awards: G. Eichhorn was invited to present a lecture at the Gordon Conference on Aging, Tilton, N. H., August, 1974.

Publications: None

Serial No. HD-GMA5
1. Gerontology Research Center
2. Laboratory of Molecular Aging
3. Molecular Chemistry Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Polymers as Biological Reagents

Previous Serial Number: Same

Principal Investigator: J. Pitha
H. Chou

Other Investigators: P. Pitha

Cooperating Units: Johns Hopkins University
Baltimore City Hospitals

Man Years:

Total:	2.08
Professional:	1.33
Others:	.75

Project Description:

Objectives: The program focuses on the effects of synthetic polymers on human cells grown in tissue culture and on the interaction of polymers with viral systems (animal viruses). The main class of macromolecules studied consists of the vinyl analogs of nucleic acids of the general formula $(\text{-CH Base - CH}_2)_n$. The ultimate objectives are to determine which synthetic polymers can be used pharmaceutically, to study viral infection in senescent cells and the possibility of its prevention by polymers, and finally, to determine whether the uptake of foreign macromolecules by cells with the age of the cells.

Methods Employed: The study requires a rather broad range of chemical and biological methods. The necessary chemical synthesis, and the in vitro work with enzymatic systems (replicase) and the uptake of dextran by cells grown in tissue culture is done at the Gerontology Research Center. The study of the replication of murine leukemia virus is done in collaboration with Dr. P. Pitha, Department of Medicine, Johns Hopkins University.

Major Findings: A. Polymers and viral replication. Polyvinyluracil and polyvinyladenine supress the replication of murine leukemia virus in cell culture and diminish the syndromes of infection by this virus in mice. To clarify the mechanism of action of the vinyl analogs, the effects of vinyl polymers on reverse transcriptase from virions were studied in detail using exogenous templates. All possible polynucleotide template - analog combinations were tested and it was concluded that the enzyme is influenced strongly only when the combinations are base complementary; that is, when there is a strong complex between the template used and the analog. The only exception to that rule is the combination of polyadenylate with polyvinyladenine, when bimodal dependence (promotion or inhibition) of incorporation exists — depending on the concentration of analog. This phenomenon was traced to the weak interaction of the analog with the template and the enzyme.

A new method was developed to study interactions between analogs and polynucleotides. When acrylamide gel is formed in the presence of vinyl polymer the latter stays practically immobile during electrophoresis and influences mobility in the gel of any nucleic acid with which it interacts; the porosity of the gel is not changed. This new method has been used to compare interactions of polyvinyladenine with polyuridylate and polyadenylate.

The effects of vinyl polymers on DNA dependent RNA polymerase from E.coli (EC 2.7.7.6) were followed; only the strong complementary interaction between the template and analog influenced the enzyme.

Tests were begun on the effects of vinyl analogs on mice and their protection from leukemia infection. Polyvinyladenine proved to have low acute toxicity to animals and decreased spleen size after infection. The fate of analog in the organism was studied with polyvinyluracil. A large portion is rapidly extracted by urine, but some is retained for a longer period of time.

B. Polymers and induction of interferon. The antiviral protein, interferon, is induced by treatment of cells in culture by the complex of polyinosinate and polycytidylate. The question as to whether there are some special receptors for this inducer on the cell membrane, and the effects of pre-treatment of cells by enzymes or polybasic compounds on induction, were studied. Induction was found to be more sensitive to the general shape of the membrane than to specific molecular damage (e.g. to proteins or polysaccharides).

C. Senescent cells. The study on uptake of foreign macromolecules (Dextran) by diploid cells in culture from medium was concluded. Foreign macromolecules somewhat shorten the lifespan of cells but are taken up approximately to the same extent by cells of intermediary or late (senescent) passages.

The study on viral infection in senescent cells have been continued using polio virus in place of the previously used vesicular stomatitis virus. Using the simpler virus with well known genesis it is hoped to elucidate the reason that cells with a diminished capacity for their own replication still manage to support viral replication normally.

Significance to Bio-medical Research and the Program of the Institute:
Synthetic compounds of high molecular weight differ profoundly from low molecular weight compounds in their interaction with living cells. They are taken up by a different mechanism, the efficiency of which seems to depend more on the growing stage of the cell than on the age of the cell. Another difference is in their degradation in cells; synthetic macromolecules have a much longer life span since they are not susceptible to the usual cellular

degradative processes. Both these differences have practical importance only when polymers with a selective biological action can be designed and synthetized. Vinyl analogs of nucleic acids do show selectivity thus pointing to their potential use as pharmaceuticals.

Proposed Course of the Project: The present results show that synthetic polymers can elicit a selective biological effect, in a manner similar to that of low molecular weight pharmaceuticals. By directed synthesis and study of the basic biological effects of polymers it is hoped to gain the knowledge necessary for the design of more potent preparations.

Honors and Awards: None

Publications:

Pitha, P. M. and Pitha, J.: Interferon Induction Site : Poly IC on Solid Substrate Carriers. J. Gen. Virol. 21:31-37 (1973).

Pitha, J., Adams, R., and Pitha, P. M.: Viral Probe into Events of Cellular (In Vitro) Aging. J. Cellular Physiol. 83:211-218 (1974).

Pitha, P. M., Harper, H. D., and Pitha, J.: Dependence of Interferon Induction on Cell Membrane Integrity. Virology, in press.

Pitha, P. M. and Pitha, J.: Interferon Induction by Polynucleotides Modified with Molecular Carriers. J. Virology, in press.

Serial No. HD-GMA9
1. Gerontology Research Center
2. Laboratory of Molecular Aging
3. Molecular Chemistry Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Relation of Structure and Function in Hemoglobin

Previous Serial Number: HD-AG-17(8)(c)

Principal Investigator: J. Rifkind

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	1.2
Professional:	0.8
Others:	0.4

Project Description:

Objectives: (1) To study the binding of ligands to hemoglobin, and the role of the protein in controlling this function. (2) To study the mechanisms for maintaining hemoglobin in its functional form. (3) To study the mechanisms involved in regulating the transport of oxygen to the tissues.

Methods Employed: Various preparative procedures are used to purify hemoglobin particularly with respect to the erythrocyte copper. Copper and other metal ion concentrations are measured by atomic absorption spectroscopy in the hemoglobin samples as well as the material separated from the hemoglobin. The oxidation of hemoglobin is investigated with and without the addition of various proteins and/or small substances. The binding of copper, zinc and other divalent metal ions to hemoglobin are studied by equilibrium dialysis. Effects of divalent metal ions on the oxidation and oxygenation of hemoglobin under various conditions are compared with the affinity of hemoglobin for the metal ions.

Major Findings: A. A possible regulatory role for a divalent metal ion binding site on hemoglobin. Our previous studies indicate that Cu(II) catalyzes the oxidation of hemoglobin. Binding studies indicate that 1 Cu(II) atom binds to every 2 subunits of hemoglobin. The relationship between the binding of Cu(II) and the oxidation is indicated by the finding that only 50% of the hemoglobin subunits are rapidly oxidized by copper as predicted by the above stoichiometry.

While Cu(II) is the only metal ion to oxidize hemoglobin, other divalent metal ions were found to bind to hemoglobin. The relative affinities of the metal ions are Cu(II) $\cong$ Zn(II) > Cd(II) > Ni(II) > Co(II). Competetive binding studies with Zn(II) and Cd(II) suggest that these metal ions probably bind the same region as Cu(II). Their failure to oxidize hemoglobin can be readily explained by the difference between the oxidation potential of copper and the other metal ions. In fact, we find that these metal ions actually reduce the rate of Cu(II) induced oxidation, which is expected if they compete with Cu(II) for the same site.

Oxidation precludes the possibility of studying oxygenation of hemoglobin in the presence of Cu(II). However, a possible role in the regulation of oxygen transport for the metal binding site is indicated by oxygenation studies in the presence of 1 Zn(II) to every 2 hemes. The Zn(II) produces a 3-fold increase in oxygen affinity which is independent of the pH. We have attempted to determine the region of the molecule where the metal ions bind, by chemically modifying the structure of hemoglobin. Thus far the binding has not been significantly affected, even though the rate of the Cu(II) induced oxidation has been decreased by certain modifications on the β-subunits.

We have also been able to grow crystals in the presence of Zn(II) in preparation for an attempt to determine the metal binding sites by x-ray crystallography.

B. The oxidation of hemoglobin. Our previous studies suggest that part of the erythrocyte copper is involved in the autoxidation of hemoglobin. Consistent with these results we have been able to remove 35% of the erythrocyte copper by dialyzing hemolyzed cells against strong complexing agents. By comparing the chromatogram on G-75 sephadex of hemolyzed cells dialyzed against complexing agents with regular hemolyzed cells, if was shown that the dialysis removes a fraction of copper which chromatographs together with hemoglobin without affecting the fraction of copper which chromatographs with lower molecular weight proteins.

While Cu(II) plays a dominant role in the oxidation of hemoglobin, other mechanism(s) seem to also be involved. This conclusion is indicated by the observation that copper only seems to catalyze the rapid oxidation of 2 of the 4 hemes and even in the presence of complexing agents a slow rate of oxidation is still observed. In the absence of copper the oxidizing agent is most probably oxygen in one or several of its oxidation states.

In a series of experiments varying the oxygen pressure and using superoxide dismutase and catalase to remove the superoxide radical and peroxide, respectively, it has been possible to show that the oxygen species responsible for the oxidation of hemoglobin is peroxide. The superoxide radical doesn't seem to affect the oxidation, and molecular oxygen actually decreases the rate of oxidation.

Significance to Bio-medical Research and to the Program of the Institute:

The physiological role of hemoglobin is to transport oxygen from the lungs to the cells. The efficient uptake and release of oxygen requires cooperative oxygen binding and the proper regulation of the oxygen affinity. It is also necessary to limit the oxidation of hemoglobin in order to maintain an adequate concentration of functional hemoglobin. These studies thus help to elucidate a vital function of organisms. The aging process can involve changes in the ability of the organism to transport oxygen to certain tissues.

Proposed Course of Project: (1) We plan to continue our studies on the various factors which influence the autoxidation of hemoglobin in the erythrocyte and in purified systems. The rapid kinetics for the copper induced oxidation of hemoglobin will be studied in order to separate the binding, conformational and electron transfer steps involved in the oxidation. (2) The divalent metal ion site on hemoglobin and its possible regulatory role will be further elucidated. The effect of various metal ions on the oxygenation of hemoglobin under different conditions will be studied. Changes in oxygenation will be correlated with binding studies on oxyhemoglobin and deoxyhemoglobin. Collaborative work with Dr. Moffat at Cornell University is being planned to locate the divalent metal binding site by x-ray techniques. The possible physiological significance of this site will be investigated. (3) We plan to study changes in the level of 2,3 DPG, Cu(II), Zn(II) and perhaps other divalent metal ions with age. This information will be combined with oxygen binding data and other hematological data in order to determine whether, and in which way, aging of the organism involves changes in the ability of the organism to transport oxygen to the tissues.

Honors and Awards: Dr. Rifkind was invited to present a seminar to the Heme-Protein group at the National Institutes of Health, Bethesda, Maryland.

Publications:

Rifkind, J. M.: The Autoxidation of Hemoglobin. II. The Dominant Role of Copper. *Biochemistry*, in press.

Serial No. HD-GMA-12
1. Gerontology Research Center
2. Laboratory of Molecular Aging
3. Section on Molecular Chemistry
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Effects of Metal Ions on the Structure and Function of Nucleic Acids

Previous Serial Number: HD-GMA1 - Interaction of Metal Ions with Polynucleotides and Related Compounds
HD-GMA2 - Degradation of Nucleic Acids by Metal Ions
HD-GMA6 - Mispairing of Nucleotide Bases
HD-AG-30 - The Function of Metal Ions in Enzymatic Reactions

Principal Investigator: G. Eichhorn, J. Butzow, P. Clark, J. Froehlich, J. Pitha, J. Rifkind, Y. Shin, R. Srivastava

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	7.4
Professional:	5.0
Others:	2.4

Project Description:

Objectives: (1) To understand the participation of metal ions in the biological activities of the nucleic acids, (2) to obtain clues to the reasons for the toxicity of metal ions, (3) to understand the possible role of metal ions in the aging process, (4) to use metal ions as probes to study the mechanism of biological information transfer.

Methods Employed: The interactions of metal ions with the nucleic acids, the nucleosides and nucleotides are studied, using a variety of physical chemical techniques, e.g. optical rotatory dispersion, circular dichroism, nuclear magnetic resonance, electron spin resonance, spectrophotometry. From these determinations binding to specific sites can be correlated with specific chemical and biological phenomena. The interaction studies are designed to answer questions about the function of the metal ions in biological systems. Metal ions are used to modify the structures of nucleic acid molecules in order to examine how these molecules are replicated and transcribed, and they are employed to bring about the selective cleavage of polynucleotides.

Major Findings: A. Platinum complexes as probes of the mechanism of RNA polymerase. Platinum complexes exist as stable geometrical isomers which form strong complexes with DNA. We have previously demonstrated the remarkably different way in which cis and trans $Pt(NH_3)_2Cl_2$ react with DNA. The difference in the behavior of these geometrical isomers can be used as a novel means of modifying the DNA template by complex formation involving the different geometries. Pt DNA templates sharply reduce the incorporation of nucleotide triphosphates during the polymerization phase of RNA synthesis by DNA-dependent RNA polymerase from E.coli. Cis and trans Pt complexes are about equally effective at high concentrations, but only the cis complex inhibits substantially at low concentrations (1 Pt/1000-4000 nucleotide). The inhibition appears to result entirely from template bound Pt; complexes had no effect on polymerization at 1000 times the enzyme concentration. RNA chain length was estimated from the ratio of ^{14}C-ATP/γ-^{32}P-ATP incorporated. The average RNA chain length decreased from 400 nucleotides per chain at Pt/DNA=1/500, to 25 at Pt/DNA=1/10. The fraction of short message not retained on the glass fiber filter increased from 15 to 50% of the total product. These experiments lead to the conclusion that the cis Pt complex at low concentration and the trans complex at higher concentration decrease the length of the message units produced, with the positions of the Pt atoms serving as the message limiting loci. The geometry of the trans complex prevents its interaction with more than one base of the same strand, but the cis complex can readily interact with two adjacent bases, thus restricting their rotation. It appears therefore that RNA polymerase activity stops whenever such a geometrically restricted base sequence is reached.

The effect of cis $Pt(NH_3)_2Cl_2$ on RNA synthesis was studied with a highly purified core enzyme which lacks the σ subunit, and a holoenzyme that contains all five subunits. When holoenzyme was challenged with fresh template, and NTPs at intervals following the addition of cis Pt DNA (1:40), progressively lower levels of activation and longer induction periods were observed. With core enzyme, however, the addition of DNA resulted in a rapid burst of activity which was not significantly altered by prolonged incubation with Pt DNA. These results can be explained by a slowly forming tight complex of Pt DNA with the holoenzyme, and a relatively weak association with the core enzyme, thus implicating the σ subunit in the strong binding.

A combination of spectrophotometric, circular dichroism, and melting studies of the reaction between the Pt complexes and DNAs of various GC contents, as well as complexes with poly dA•poly dT and poly dG•poly dC have revealed that Pt binds predominately to the GC region of DNA. Poly dA•poly dT is virtually unaffected by Pt complexes. Hydrolysis of Pt-DNA complex by DNAse, and examination of hydrolysis products indicates that Pt is strongly bound to guanine residues.

B. The effect of metal ions on the conformation of polynucleotides. The thermally induced conformational transitions of single stranded stacked polynucleotides are noncooperative; i.e., the ordered structure of the molecule breaks down gradually. Copper(II) ions make these transitions highly cooperative; i.e., once the ordered structure has been disturbed anywhere, it breaks down everywhere at once.

The binding of Cu(II) to polynucleotides is a complex process that involves both noncooperative and cooperative processes. A method was developed to analyze the binding curves to determine the relative significance of these two processes, and to estimate noncooperative and cooperative binding constants. At low copper concentrations noncooperative binding, presumably to phosphate, predominates. This binding has essentially no effect on optical properties in the presence of salt. In the absence of added salt changes are observed which indicate increased order. At higher copper concentrations, cooperative copper binding is observed, and it is this binding which is responsible for the disordering of the polynucleotides.

Copper(II) ions do not affect the conformation of poly d(AT), although they bind effectively to the component bases. Apparently copper coordination is unable to compete with the hydrogen bonding of the complementary bases in poly d(AT). Silver(I) and platinum(II) have small effects on the d(AT) conformation, but mercury(II) has very profound effects. These phenomena are useful for the detection of AT-rich regions of DNA.

C. Degradation of nucleic acids by metal ions. 28S ribosomal RNA labelled with I-125 was subjected to degradation by excess zinc (10 Zn/nucleotide) at 40°, and the fragments separated by electrophoresis through polyacrylamide gel and detected by radioactive counting. Large fragments are produced early in the reaction, indicating that the convoluted structure of such long chain RNA can serve to limit zinc cleavage just as in 5-S RNA. Thus zinc cleavage may be a useful method for producing large, defined fragments from long-chain RNA.

It has now been established that beef pancreatic deoxyribonuclease I, the most frequently used enzyme for splitting DNA, generally splits the phosphodiester bond on only one strand adjacent to a given base pair, regardless of the metal ion used to activate the enzyme. These results are in contradiction to the findings of the previously used more indirect methods, such as viscosity measurements. These measurements can be interpreted in a variety of ways. The present scheme involves the determination of the presence or absence of complementarity of the bases in the DNA fragments, and the observation of complementarity is a direct indication of the simultaneous cleavage of both DNA chains. In all instances non-complementarity of bases was observed.

D. Metal ions in tissue culture, and effects of age. Initial studies on the effects of metal ions on (WI-38) cells grown in tissue culture indicate that the concentration of some metal ions is higher in "old" cells than in "young". This study has led to an explanation of the large variety of results that can be found in the literature on metal content of tissues, and the difficulties of making correlations. Each metal ion has a characteristic elution curve when the cells are treated with washing media, and comparisons at different points on this curve are obviously invalid. Through analysis of various points on the elution curve it is possible to obtain results for strongly bound and weakly bound metals.

Significance to Bio-medical Research and to the Program of the Institute: The participation of metal ions in every aspect of genetic information transfer makes the study of the interaction of metal ions with the nucleic acids of major importance in understanding the perturbation of the genetic information that is presumably characteristic of the aging process.

Proposed Course of Project: It is intended to continue studies to determine the sites of binding of metal ions on nucleic acids, the conformational changes induced by such binding, and to correlate these effects with biological function. We shall continue to evaluate age changes in the metal content of WI-38 cells, with the eventual goal of establishing the impact of these changes on cellular biochemistry. Transient kinetic studies on the Ca^{2+} - Mg^{2+} ATPase solubilized from skeletal sarcoplasmic reticulum will be carried out; a specially designed apparatus will be used to follow pre-steady state phosphorylation and phosphate release with the purpose of defining the detailed enzymatic steps, and to compare the reconstituted system with the intact system.

Honors and Awards:

G. Eichhorn was invited to present a plenary lecture at the NATO Conference on Eco-toxicity of Heavy Metals and Halogen Compounds at Mont Gabriel, Canada, May, 1974.

G. Eichhorn was invited as one of the lecturers at the NATO Advanced Study Institute on Ligand Reactivity of Coordination Compounds and Homogeneous Catalysis, Corfu, Greece, June, 1974.

G. Eichhorn was invited to present a Main Lecture at the 16th International Conference on Coordination Chemistry, Dublin, Ireland, August, 1974.

G. Eichhorn was invited to participate in the International Conference on Biology and the Future of Mankind, Paris, France, September, 1974.

G. Eichhorn was invited to present lectures at various universities.

Publications:

Eichhorn, G. L. (Ed.): Inorganic Biochemistry, Vol. II, Elsevier Publishing Co., Amsterdam, 1973, 691 pp.

Eichhorn, G. L.: Complexes of Nucleosides and Nucleotides. In Eichhorn, G.L. (ed.): Inorganic Biochemistry. Amsterdam, Elsevier Publishing Co., 1973, pp. 1191-1209.

Eichhorn, G. L.: Complexes of Polynucleotides and Nucleic Acids. In Eichhorn, G.L. (ed.): Inorganic Biochemistry. Amsterdam, Elsevier Publishing Co., 1973, pp. 1210-1243.

Eichhorn, G. L., Berger, N. A., Butzow, J. J., Clark, P., Heim, J., Pitha, J., Richardson, C., Rifkind, J. M., Shin, Y., and Tarien, E.: Some Effects of Metal Ions on the Structure and Function of Nucleic Acids. In Dhar, S.K. (ed.): Metal Ions in Biological Systems. New York, Plenum Publishing Co., 1973, pp. 43-66.

Shin, Yong Ae: Interaction of Metal Ions with Polynucleotides and Related Compounds. XXII. Effect of Divalent Metal Ions on the Conformational Changes of Polyribonucleotides. Biopolymers 12: 2459-2475, 1973.

Hall, W. K., Henry, P., Benkovic, S. J., Bjerrum, J., Bray, R. C., Eichhorn, G. L., Eley, D. D., Fripiat, J. J., Knözinger, H., Maitlis, P. M., and Rigo, A.: Kinetics and Mechanisms. In Basolo, F., and Burwell, R. J. Jr. (Eds.): Catalysis, Progress in Research. London and New York, Plenum Press, 1973, p. 131-140.

Srivastava, R. C., Froehlich, J., and Eichhorn, G. L.: The Effect of Platinum Complexes on the Structure and Function of DNA. Circular Dichroism, Continuous Variation, and Enzymatic Studies. In Connors, T. A., and Roberts, J. J. (Eds.): Platinum Coordination Complexes in Cancer Chemotherapy. Heidelberg, Springer-Verlag, in press.

Serial No. HD-GMA-13
1. Gerontology Research Center
2. Laboratory of Molecular Aging
3. Section on Intermediary Metabolism
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Control of Intermediary Metabolism: Biology of Membranes

Previous Serial Numbers: HD-GMA 7, Cellular Regulation of Intermediary Metabolism; HD-GMA 8, Investigations on the Biochemical Mechanisms of Hormonal Regulation of Metabolism; HD-AG23(8), Mechanisms of Membrane and Cellular Transport

Principal Investigator: Bertram Sacktor, Ph.D.

Other Investigators: Bernard A. Bulos, Ph.D.; Richard G. Hansford, Ph.D.; Paul A. Insel, M.D.; Charles R. Filburn, Ph.D.; Peter S. Aronson, M.D.; Edward R. George, M.D.; Steven J. Fass, M.D.; Peter K. Chiang, Ph.D; Roger N. Johnson, Ph.D.

Cooperating Units: Department of Physiology, University of Pennsylvania; Johnson Research Foundation for Medical Physics, University of Pennsylvania; Max-Planck Institute for Biochemistry, Munchen, Germany-W

Man Years:

Total:	19
Professional:	10
Other:	9

Project Description:

Objectives: The thrust of our research on the mechanisms by which metabolic processes are regulated is directed towards what we conceive may be the "next frontier" in biomedical research — membrane biology. The problems and questions studied relate to:

1. The molecular organization of the membrane.
2. The role of the membrane in vectorial transport of small molecular species and protonmotive force.
3. The membranal reception of hormonal stimuli, the transduction and propagation of these stimuli.
4. The catalytic and integrative function of membranes:
5. The turnover of membranes.
6. The failure to maintain membrane structure, leading to cell death.

The fundamental information developed by these studies is essential to and is applicable to the understanding of the biological processes whereby physiological control systems become modified during development and aging.

Methods Employed: Three membrane systems have been employed as models. These are (1) the apical region of the plasma membrane of the rabbit renal proximal tubule, the renal brush border; (2) the biochemically and physiologically uniquely active mitochondria from blowfly flight muscle; and (3) mitochondria isolated from the rabbit heart.

A great variety of techniques are employed as the research encompasses many specialties. New methods developed and adapted include: isolation of specific, highly purified, subcellular components; electron microscopy and histochemical procedures; enzyme activity and metabolite analyses; computer programing of kinetic models; combinations of column chromatographic and enzymatic methods for the measurements of cyclic nucleotide hydrolyses at physiological levels of substrate; and measurements of binding capacity to membranes and transport into membrane vesicles.

Major Findings: I. Transport of D-glucose by Renal Membranes.

The uptake of D-glucose into vesicles of membranes isolated from the apical region of the plasma membrane of the renal cortical proximal tubule was shown to be a Na^+-gradient dependent facilitated transport process. In the brush border system Na^+ gradients were artificially imposed, whereas in more intact systems it is the ouabain sensitive active extrusion of Na^+ across the lateral and basal membranes of the tubule cell that presumably establishes the gradient required for uphill sugar transport. The initial rate of D-glucose (1 mM) transport into the membrane vesicles was stimulated 7-fold by the Na^+ gradient. At lower concentrations of sugar (50 μM) transport was increased 35- to 40-fold by the Na^+ gradient. The concentration of Na^+ required for half-maximal stimulation was 30 mM. Within the range of D-glucose concentration from 1-10 mM, Na^+ stimulated uptake by an increase in the Vmax from 2-3 to 32 nmoles/min/mg protein and by a reduction in the apparent Km for transport from 12 to 2 mM. The uptake of D-glucose in the presence of an initial Na^+ gradient showed an overshoot above the equilibrium value with ultimate return to the equilibrium value as the Na^+ gradient was dissipated during the incubation. Neither Li^+, K^+ nor choline ions caused augmentation of transport. The patterns of inhibition of the Na^+ stimulated transport of D-glucose by sugar analogs, including L-glucose, galactose, α-methyl glucoside, 2-deoxyglucose, 3-o-methylglucose and mannose, and phloridzin closely resembled known physiological data. These findings are consistent with and provide the first strong experimental support in renal membrane systems for the Na^+ gradient uphill transport of D-glucose hypothesized by Crane.

II. Transport of Amino Acids by Renal Membranes.

The transport of amino acids across isolated plasma membranes of mammalian cells has not been reported previously. A study of the mechanisms and specificity of amino acid uptake by the renal brush border was initiated both (1) as a general model for membrane transport, which has important implications not only on renal physiology but on the transport of amino acids in the intestine and choroid plexus, and (2) as a significant specific model, which bears directly on understanding amino aciduric conditions stemming from defects in transport.

For the first time, the uptake of an amino acid (alanine) by the isolated renal luminal membrane was shown to be consistent with a concept for a Na^+ gradient dependent facilitated transport system. The initial rate of alanine transport into the membrane vesicles was stimulated about 3-fold by a Na^+ gradient. In the presence of the gradient the uptake was saturable with time and concentration, in the µmolar range. Other cations, including K^+, Li^+ and choline were ineffective.

The enzyme γ-glutamyl transpeptidase was proposed recently as the amino acid carrier. The transpeptidase was found to be enriched 10-fold in the purified renal brush border preparation, relative to the cortex homogenate. Thus, the enzyme, whose relative specific activity is increased to almost that of trehalase, is an excellent enzyme marker for the brush border membrane. Na^+ was also found to enhance γ-glutamyl transpeptidase activity.

III. Interactions of Hormones with Membranes and the Regulation of Metabolism.

Investigations of hormonal regulation of metabolism were focused on the mechanisms of endocrine control of renal transport. Specific questions dealt with: (1) the localization in the renal cortex of the membrane bound hormonally stimulated adenyl cyclase; (2) the binding of cAMP and cGMP to luminal membranes; (3) the hydrolysis of cyclic nucleotides by membranal phosphodiesterase; (4) the identification and characterization of the membrane bound protein kinase; (5) the identification of the endogenous protein in the membrane that is phosphorylated; and (6) how the presumed phosphorylated-dephosphorylated protein acts to control the transports of Pi, Ca^{2+} and, perhaps Na^+.

The specific activities of renal cortical adenyl cyclase, basal as well as that activated by NaF, catecholamines and parathyroid hormone, were not elevated significantly in the brush border membrane relative to those in other membranes. This finding suggests that in the renal cortex cAMP is generated on the anti-luminal regions of the plasma membrane. However, since cAMP must traverse the cell to interact with the luminal membrane, *e.g.*, in the action of parathyroid hormone on Pi transport, and in the appearance of cAMP in the urine, the binding of cAMP to the brush border membrane was characterized. Binding was saturable, approximately 1 pmole/mg protein was bound at 20 nM cAMP, with multiple binding sites. Binding was reversible. Bound cAMP was resistant to hydrolysis by endogenous or exogenous phosphodiesterase. Binding was unaffected by pH, Na^+ gradients, hormones and drugs including: parathyroid hormone, calcitonin, vasopressin, prostaglandins, isoproteranol, bradykinin, probenecin and cholecalciferol. Binding was markedly inhibited by sulfhydryl reagents (partially reversed by thiols). cIMP, dibutyryl cAMP and cGMP inhibited cAMP binding. The molar ratios of 50% inhibition of the binding of 20 nM cAMP were: cAMP - 1.5, cIMP - 30, dibutyryl cAMP - 300, cGMP - 1300. cGMP was bound at a rate about 0.1 that of cAMP. The binding and reversal of binding of cGMP have different temporal and concentration kinetics from those of cAMP.

The levels of cyclic nucleotides at the luminal membrane region of the tubular cell could be controlled by brush border phosphodiesterases. Enzymes hydrolyzing both cAMP and cGMP were evident. At least two enzymes acting on cAMP were found. Phosphodiesterase I was maximally active at pH 8, stable at 53°, apparent Km for cAMP $>10^{-3}$ M, and, upon EGTA inhibition, reactivated by Ca^{2+} but not by Mg^{2+}. Phosphodiesterase II was maximally active at pH 6-7, labile at 53°, and, upon EDTA inhibition, reactivated by Mg^{2+} but not Ca^{2+}. Cobalt activated both chelator-inhibited enzymes. Less activity was found for cGMP as substrate than for cAMP. For example, at pH 8 and high substrate concentrations, the Vmax for cAMP and cGMP hydrolysis were 1.4 and 0.5 µmoles/hr/mg, respectively.

Protein kinase activity was demonstrated in which the brush border membrane acted as both enzyme and substrate. The apparent Km for ATP was 62 µM and activity was Mg^{2+} dependent (10 mM optimal). Characterization of the lability and stability of the product of the reaction, protein-PO_4, indicates a phospho-ester bond rather than an acyl-phosphate. The ability of the membrane to phosphorylate itself may be important in control of proximal tubular function.

IV. The Catalytic and Integrative Function of Mitochondrial Membranes.

The role of the membrane in the control of mitochondrial oxidations and, indeed, of the basic mechanisms of energy transduction was studied. These investigations were centered on the control of the complete oxidation of pyruvate, a process involving the operation of both pyruvate dehydrogenase and the entire tricarboxylate cycle, and involved the two main approaches to the regulation of intermediary metabolism: (1) the study of enzyme kinetics in vitro; and (2) the variations in the steady state concentrations of key intermediates upon altering the flux through the metabolic pathway.

The control of pyruvate dehydrogenase activity by enzymatic phosphorylation and dephosphorylation was studied in intact mitochondria isolated from rabbit heart. It was demonstrated that pyruvate dehydrogenase could be completely inactivated by incubating mitochondria with ATP, oligomycin and NaF, and that this loss in dehydrogenase activity was correlated with the incorporation of ^{32}P from $[\gamma\text{-}^{32}P]$ATP into mitochondrial protein(s) and with a decrease in the mitochondrial oxidation of pyruvate via the tricarboxylate cycle. The ATP may be supplied exogenously, generated from endogenous ADP during oxidative phosphorylation, or formed from exogenous ADP in FCCP-uncoupled mitochondria. With coupled mitochondria the concentration of added ATP required to half-inactivate the dehydrogenase was 0.24 mM. With uncoupled mitochondria the apparent Km was decreased to 60 µM ATP, a value approaching that obtained with the solubilized purified kinase. The inactivation of pyruvate dehydrogenase in intact mitochondria by exogenous ATP was sensitive to atractyloside, suggesting that pyruvate dehydrogenase kinase acts internally to the atractyloside-sensitive barrier. The divalent cation ionophore, A23187, enhanced the loss of dehydrogenase activity. In the intact mitochondria A23187 may decrease dehydrogenase activity by inhibiting both ATPase and pyruvate dehydrogenase phosphatase activities. In addition to divalent cations, the expression of pyruvate dehydrogenase kinase activity in intact cardiac mitochondria is regulated by pyruvate, inorganic phosphate and ADP.

Pyruvate, in the presence of rotenone, strongly inhibited kinase activity. This suggests that pyruvate facilitates its own oxidation and that increases in pyruvate dehydrogenase activity by substrate may provide a modulating influence of the utilization of pyruvate via the tricarboxylate cycle. Inorganic phosphate protected the dehydrogenase from inactivation by ATP. ADP added to the incubation mixture together with ATP, inhibited the inactivation of pyruvate dehydrogenase. This protection may result from a direct action on the kinase, as ADP competes with ATP, and an indirect action, in that ADP competes with ATP for the translocase. It is suggested that the intramitochondrial [ATP]/[ADP] ratio effects pyruvate dehydrogenase kinase activity directly whereas the cytosolic [ATP]/[ADP] ratio acts indirectly.

The inactive phosphorylated pyruvate dehydrogenase in intact cardiac mitochondria was completely activated by dephosphorylation by a specific pyruvate dehydrogenase phosphatase. The phosphatase was inhibited by NaF and divalent cation chelators. The phosphatase required Mg^{2+}, at mM concentrations. Reactivation of the dehydrogenase was extremely sensitive to Ca^{2+}, only 10^{-6} to 10^{-7} M was needed for full activity.

Blowfly flight muscle mitochondria may be the ideal experimental material for a study of the control of mitochondrial oxidations and, indeed, of the basic mechanisms of energy transduction. This is because the oxidations of the blowfly flight muscle are the most active and the most controlled known in biology. In addition, mitochondria from this tissue are highly adapted to the simple role of synthesizing ATP, and, lacking the ancillary functions found in mitochondria from other tissues which are metabolically more versatile, also lack enzymes, metabolites and specific permeabilities found in all other mitochondria. In short, blowfly flight muscle mitochondria present a model system for the study of the process of oxidative phosphorylation and its control.

To pinpoint which of the several reactions of the tricarboxylate cycle is the locus of control in blowfly flight muscle, the steady state concentrations of citrate, isocitrate, α-ketoglutarate, malate, CoA, acetyl CoA and succinyl CoA were measured in mitochondria during the transition from State 4 (respiration limited by ADP) to State 3 (fully active). On activating respiration with ADP, the mitochondrial contents of citrate, isocitrate and acetyl CoA decreased whereas those of α-ketoglutarate, succinyl CoA, malate and CoA increased. Measurements of the tri- and dicarboxylates in the flight muscle of blowflies *in vivo* before and after initiation of flight were entirely parallel to those found on the activation of mitochondrial metabolism *in vitro*. These changes, combined with the knowledge that the reaction catalyzed by isocitrate dehydrogenase is not at equilibrium, allowed the unequivocal identification of this enzyme as the locus of control in this tissue.

It was found previously that the activity of isocitrate dehydrogenase was inhibited by Ca^{2+}. Thus, Ca^{2+} could effectively function to control the complete oxidation of pyruvate via the tricarboxylate cycle. A kinetic study was carried out on the inhibition of pyruvate oxidation by Ca^{2+}. Pyruvate oxidation was inhibited almost completely by physiological levels of Ca^{2+}; the Ki was 10 µM. The kinetic nature of the inhibition with respect to components of the system, including ADP, Pi and pyruvate, was determined.

The kinetic properties of succinic thiokinase from flight muscle were studied because of a previous suggestion that this enzyme may be a locus of control and because the enzyme was found to have properties very different from those described from other animal sources. The blowfly enzyme was totally specific for adenine nucleotides, in contradistinction to a requirement for guanosine nucleotides with other preparations. Apparent Km values for substrates succinyl CoA, ADP and Pi were determined separately at saturating concentrations of co-substrates. The same was determined for the substrates of the reverse reaction, succinate, ATP and CoA. Studies in which the composition of a mixture of ADP and ATP was varied, while the total concentration remained constant, as would be the case in intact mitochondria, indicate that this enzyme could not be the locus of control of pyruvate oxidation, and this was proved by the metabolite assays reported above.

Steady state concentrations of intramitochondrial NAD and NADH in flight muscle were measured in order to determine whether the transfer of electrons to the respiratory chain was rate-limiting relative to the transfer of electrons from the respiratory chain to O_2. Intramitochondrial NADH was found to rise on adding ADP to mitochondria oxidizing pyruvate. Experiments in which NAD and NADH were isolated from flight muscle showed that there was a parallel rise in NADH content on initiation of flight. Thus, these results obtained both from the intact insect, in vivo, and from isolated mitochondria, in vitro, tend to indicate that the activity of the dehydrogenases increase relative to that of electron transport and phosphorylation, on imposition of an energy demand upon the mitochondria. This concept is completely contrary to all other hypotheses on mitochondrial bioenergetics and will be investigated further.

Significance to Bio-medical Research and the Program of the Institute: The fundamental information developed by these studies on the biology of membranes is essential to and is applicable to the understanding of the biological processes whereby physiological control systems become modified during development and aging.

Proposed Course: Specific new questions to be studied relate to and include: 1. In the ion-gradient model of transport in the kidney, whether the Na^+-coupled glucose transfer is an electroneutral or an electrogenic process. 2. The specificities of the amino acid transport systems. 3. The mechanism of phosphate transport in the proximal tubule. 4. Identification of the endogenous proteins in the membrane that are phosphorylated or dephosphorylated by hormonally stimulated protein kinase and phosphatase. 5. The mechanism by which Ca^{2+} inhibits isocitrate dehydrogenase. 6. The control of phosphofructokinase, the rate limiting enzyme in glycolysis. 7. The determination of whether mitochondrial oxidation is limited at the dehydrogenase or respiratory chain level.

Honors and Awards:

Dr. Sacktor was an invited symposium speaker at the Annual Meeting of the Entomological Society of America, Dallas, Texas.

Dr. Sacktor was a visiting professor and external examiner to the Department of Biology, University of Western Ontario, London, Ontario, Canada.

Dr. Insel was an invited speaker at Vanderbilt University, School of Medicine, Department of Medicine, Section of Metabolism and Clinical Pharmacology.

Dr. Insel was an invited speaker at the University of California, San Francisco School of Medicine, Division of Clinical Pharmacology.

Dr. Insel became Diplomate, American Board of Internal Medicine.

Dr. Aronson was an invited speaker to the Department of Medicine, Yale University.

Dr. Aronson became Diplomate, American Board of Internal Medicine.

Dr. Hansford was an invited speaker to the Department of Biophysics, Johnson Foundation, University of Pennsylvania.

Publications:

Aronson, P. S., and Sacktor, B.: Transport of D-glucose by brush border membranes isolated from the renal cortex. *Biochim. Biophys. Acta.* In press.

Chesney, R., Sacktor, B., and Kleinzeller, A.: The binding of phloridzin to the isolated luminal membrane of the renal proximal tubule. *Biochim. Biophys. Acta* 332: 263-277, 1974.

Coe, E. L., and Greenhouse, W. V.V.: Possible regulatory interactions between compartmentalized glycolytic systems during initiation of glycolysis in ascites. *Biochim. Biophys. Acta* 329: 171-182, 1973.

Gillespie, D., Jacobson, A., Gillespie, S., and Patterson, D.: Use of exhaustive nucleic acid hybridization for determining the amount and size distribution of newly synthesized bacterial message RNA. *Biochem. Genet.* 10: 175-190, 1973.

Greenhouse, W. V.V., and Coe, E. L.: The effect of K^+ depletion on the initial kinetics of glycolysis in ascites tumor cells. *Biochim. Biophys. Acta* 329: 183-195, 1973.

Hansford, R. G.: The control of tricarboxylate cycle oxidations in blowfly flight muscle. The steady state concentrations of coenzyme A, acetyl coenzyme A and succinyl coenzyme A in flight muscle and isolated mitochondria. *Biochemical Journal.* In press.

Hochman, M. S., and Sacktor, B.: Brain mitochondrial hexokinase: solubilization by adenine nucleotides and in coupled respiration. *Biochem. Biophys. Res. Commun.* 54: 1546-1553, 1973.

Hochman, M. S., Shimada, Y., and Sacktor, B.: Solubilization of brain mitochondrial hexokinase by ATP and glucose-6-phosphate: ultrastructural aspects. *J. Neurochem.* In press.

O'Brien, S. J.: Comparative analysis of malate dehydrogenases of *Drosophila melanogaster*. *Biochem. Genet.* 10: 191-205, 1973.

O'Brien, S. J., and Gethmann, R. C.: Segmental aneuploidy as a probe for structural genes in *Drosophila*: mitochondrial membrane enzymes. *Genetics* 75: 155-167, 1973.

Sacktor, B.: Biochemistry of insect flight. Part I: Utilization of fuels by muscle. Candy, D. J., and Kilby, B. A. (eds.): *Insect Biochemistry*. Chapman & Hall Ltd., London, In press.

Sacktor, B.: Biological oxidations and energetics in insect mitochondria. Rockstein, M. (ed.): *Physiology of Insecta IV*, Chapter 5, 271-353. Academic Press, New York and London. In press.

Sacktor, B., Chesney, R. W., Mitchell, M. E., and Aronson, P. S.: The interactions of D-glucose with the renal brush border. Fanelli, G. M., and Wesson, L. G. (eds.): *Proceedings of the A. N. Richards Symposium on* Recent Advances in Renal Physiology and Pharmacology. University Park Press, Baltimore. In press.

Sacktor, B., Wu, N-C., Lescure, O., and Reed, W. D.: Regulation of muscle phosphorylase *b* kinase activity by inorganic phosphate and calcium. *Biochem. J.* 137: 535-542, 1974.

Sussman, M. D.: Aging of connective tissue. Physical characteristics of healing wounds in young and old rats. *Am. J. Physiol.* 224: 1167-1171, 1973.

National In[illegible] of Health
Bethesda, Maryland 20014

DATE DUE

GAYLORD
PRINTED IN U.S.A.

LI
NIH
Amazing Research.
Amazing Help.

http://nihlibrary.nih.gov

10 Center Drive
Bethesda, MD 20892-1150
301-496-1080

NIH LIBRARY
4 0130 0510

WERT
OKBINDING
DDLETOWN, PA.
CEMBER 74
e Quality Bound

NIH LIBRARY
3 1496 00195 1360